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Reactivity of Murine Cytokine Fusion Toxin, Diphtheria Toxin$_{390}$-Murine Interleukin-3 (DT$_{390}$-mIL-3), With Bone Marrow Progenitor Cells

By Chung-Huang Chan, Bruce R. Blazar, Lawrence Greenfield, Robert J. Kreitman, and Daniel A. Vallera

Myeloid leukemias can express interleukin-3 receptors (IL-3R). Therefore, as an antileukemia drug, a fusion immunotoxin was synthesized consisting of the murine IL-3 (mIL-3) gene spliced to a truncated form of the diphtheria toxin (DT$_{390}$) gene coding for a molecule that retained full enzymatic activity, but excluded the native binding domain. The DT$_{390}$-mIL-3 hybrid gene was cloned into a vector under the control of an inducible promoter. The fusion protein was expressed in *Escherichia coli* and then purified from inclusion bodies. The fusion toxin was potent because it inhibited FDC-P1, an IL-3R–expressing murine myelomonocytic tumor line (IC$_{50}$ = 0.025 nmol/L or 1.5 ng/mL). Kinetics were rapid and cell-free studies showed that DT$_{390}$-mIL-3 was as toxic as native DT. DT$_{390}$-mIL-3 was selective because anti–mIL-3 monoclonal antibody, but not irrelevant antibody, inhibited its ability to kill. Cell lines not expressing IL-3R were not inhibited by the fusion protein. Because the use of DT$_{390}$-mIL-3 as an antileukemia agent could be restricted by its reactivity with committed and/or primitive progenitor cells, bone marrow (BM) progenitor assays were performed. DT$_{390}$-mIL-3 selectively inhibited committed BM progenitor cells as measured by in vitro colony-forming unit–granulocyte-macrophage and in vivo colony-forming unit-spleen colony assays. To determine if this fusion protein was reactive against BM progenitor cells required to rescue lethally irradiated recipients, adoptive transfer experiments were performed. Eight million DT$_{390}$-mIL-3–treated C57BL/6 Ly5.2 BM cells, but not 4 million, were able to rescue lethally irradiated congenic C57BL/6 Ly5.1 recipients, suggesting that progenitor cells might be heterogenous in their expression of IL-3R. This idea was supported in competitive repopulation experiments in which DT$_{390}$-mIL-3–treated C57BL/6 Ly5.2 BM cells were mixed with nontreated C57BL/6 Ly5.1 BM cells and used to reconstitute C57BL/6 Ly5.1 mice. A significant reduction, but not elimination, of Ly5.2-expressing cells 95 days post-BM transplantation and secondary transfer experiments indicated that IL-3R is not uniformly expressed on all primitive progenitor cells. The fact that some early progenitor cells survived DT$_{390}$-mIL-3 treatment indicates that this fusion toxin may be useful in the treatment of myeloid leukemias that express the IL-3R.

INTERLEUKIN-3 (IL-3) is generally considered a cytokine with a broad spectrum of activities on different hematopoietic cells at various stages of development.[1,2] This hematopoietic cytokine is also associated with leukemia. Murine and human myeloid and lymphoid leukemic cells display receptors for IL-3, and many have been shown to be responsive to exogenous IL-3 in vitro.[3,4] Production of IL-3 has been shown in some transformed myeloid cells, raising the possibility that autocrine production of IL-3 may contribute to their transformed phenotype.[5,6] In fact, neutralizing anti–IL-3 and anti–IL-3 receptor (anti–IL-3R) antibodies inhibit the growth of IL-3–producing transformed cell lines.[7,8]

In normal hematopoiesis, it is controversial as to whether there are IL-3R expressed on primitive progenitor cells.[9-11] It is known that IL-3 supports the proliferation and terminal differentiation of multipotential and committed myeloid progenitors[12] and the activation of a variety of mature myeloid cells.[13-15] IL-3 also has proliferative effects on CD10$^+$ progenitor B cells, mature tonsillar B cells, plasma-cell precursors, and CD4$^-$8$^-\alpha\beta^+$ T cells.[16-19]

To study the expression of IL-3R on primitive progenitor cells and perhaps devise a new antileukemia agent, we synthesized an IL-3 fusion toxin protein. The murine IL-3 (mIL-3) gene has been cloned and genetically mapped to chromosome 11.[20,21] It consists of 5 exons and encodes a secretory peptide with 140 residues.[22] Receptor expression is a prerequisite for response to a cytokine. The high-affinity IL-3R is composed of α and β subunits.[23] The binding of IL-3 to its receptor causes rapid internalization of the ligand-receptor complex.[24] Because of the internalization of IL-3, we reasoned that IL-3 could serve as a ligand for delivering a toxic molecule such as diphtheria toxin (DT) to the cells bearing the IL-3R.

DT is a well-studied glycoprotein with a molecular weight of 58 kD. DT has potent cell killing ability through ADP-ribosylation of elongation factor-2, resulting in inhibition of cellular protein synthesis and death of the cell. Delivering a single DT molecule into the cytoplasm is sufficient to kill a cell.[25] Native DT contains three domains: the cell binding domain, the translocation domain, and the enzymatic cytotoxic domain.[26,27] The cell-binding domain of the DT gene can be replaced by a growth factor gene, resulting in a toxin-growth factor hybrid gene, whose protein product is targeted to a specific growth factor receptor.[28-33]

The goal of these studies was to determine whether DT$_{390}$-mIL-3 could be used as an antileukemia agent and whether

From the Department of Therapeutic Radiology, Section on Experimental Cancer Immunology, the Department of Laboratory Medicine and Pathology, the Department of Pediatrics, Division of Bone Marrow Transplantation, University of Minnesota Hospital and Clinics, Minneapolis, MN; Roche Molecular Sytems, Alameda, CA; and The National Cancer Institute, Bethesda, MD.

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Address reprint requests to Daniel A. Vallera, PhD, Box 367 UMHC, Harvard Street at East River Road, Minneapolis, MN 55455.

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Table 1. Primers Used in These Studies

(a) 5'AGATATA*CCATGG*GCGCTGATGATGTTGTTGAT3'
Nco I--------DT-------- -
(b) 5'CCGGCCACTGATTGAAGC AAATGGTTGCGTTTT3'
--------IL-3------ ------DT------ -
(c) 5'AAAACGCAACCATTT GCTTCAATCAGTGGCCGG3'
-------DT------ --------IL-3-------
(d) 5'TGCCT*TGATCA*TTAATGATGATGATGATGATG ACATTCCACGGTTCCACG3'
-*Bcl* I- ------His.Tag------------IL-3-------

its use would be restricted. Specifically, it was important to determine whether it would entirely eliminate committed and primitive progenitor cells and subsequently prevent bone marrow (BM) recovery in adoptive BM transfer and competitive repopulation experiments. This would provide further insight as to the expression of IL-3R on progenitor cells and indicate further potential of DT_{390}-mIL-3 as an antileukemia agent.

MATERIALS AND METHODS

Construction of hybrid gene and plasmid. The hybrid gene encoding DT_{390}-mIL-3 was constructed by the method of gene splicing by overlap extension (SOE), as described.[34] Oligonucleotide primers were synthesized using cyanomethyl phosphoramidite chemistry on an Applied Biosystems model 380 A DNA synthesizer and purified by chromatography on Oligonucleotide Purification Cartridges (Applied Biosystems Inc, Foster City, CA) as recommended by the manufacturer. Purified oligonucleotides were resuspended in TE buffer (10 mmol/L Tris base, 1 mmol/L EDTA, pH 8.0). The primers used in these studies are given in Table 1.

Briefly, a DT gene fragment was generated in the first polymerase chain reaction (PCR) by using 5.5 ng plasmid containing the cDNA of DT mutant cross-reacting material (CRM107) as a template with primers a and b. Primer a created an *Nco* I restriction site, an ATG initiation codon, and the coding sequence of the first 7 amino acids of the DT molecule. Primer b introduced a coding sequence of amino acids 385 to 389 of the mature DT and that of amino acids 27 to 32 of the murine IL-3 molecule. A murine IL-3 gene fragment was generated in the second PCR by using 2.74 ng plasmid containing the cDNA of murine IL-3 as a template with primers c and d. Primer c created sequence homology with the 3′ end of the DT fragment generated in the first PCR. This region of homology was placed 5′ to the sequence encoding amino acids 27 to 32 of the IL-3 molecule. Primer d introduced a *Bcl* I restriction site and a TAA stop codon at the end of the IL-3 molecule. The two fragments generated in the PCRs described above were then purified and used as templates in an SOE reaction using primers a and d. This SOE formed the full-length DT_{390}-mIL-3 hybrid gene that was digested with restriction enzymes *Nco* I and *Bcl* I (GIBCO BRL, Gaithersburg, MD) and ligated into the *Nco* I and *Bam*HI cloning sites in the pET11d plasmid (Novagen, Madison, WI). The assembly of plasmid pDT-IL-3 is shown in Fig 1.

DT_{390}-mIL-4 and DT_{390}-hIL-2 were made from hybrid genes by gene splicing by SOE, as described.[33] The plasmids were assembled with the same orientation as the DT_{390}-mIL-3 plasmid. For DT_{390}-mIL-4, the final protein contained amino acids 25 through 144 of the mature IL-4 molecule. For DT_{390}-mIL-2, the final protein contained amino acids 3 through 135 of the mature IL-2 molecule.

Expression and localization of fusion proteins. Plasmid, pDT-IL-3 was transformed into the *Escherichia coli* strain BL21(DE3) (Novagen) and protein expression was evaluated. Briefly, recombinant bacteria were grown in superbroth (32 g/L bacto-tryptone, 20 g/L bacto-yeast extract [Difco, Detroit, MI], and 5 g/L NaCl, pH 7.5) supplemented with 0.5% glucose, 1.6 mmol/L $MgSO_4$, and 100 μg/mL carbenicillin (Sigma, St Louis, MO) at 37°C. When the absorbency (A_{650}) of the culture reached 1.0, expression of the hybrid gene was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG; GIBCO BRL). Ninety minutes after induction, the bacteria were harvested by centrifugation at 5,000*g* for 10 minutes. To determine the localization of expressed protein, an aliquot of bacterial pellet was resuspended in 30 mmol/L Tris, pH 7.5, 20% sucrose, 1 mmol/L EDTA and osmotically shocked by placing in ice-cold 5 mmol/L $MgSO_4$. The periplasmic fraction (supernatant) was obtained by centrifugation at 8,000*g* for 10 minutes. Another aliquot of bacterial pellet was resuspended in sonication buffer (50 mmol/L sodium phosphate, pH 7.8, 300 mmol/L NaCl). After incubation at −20°C for 16 hours, the resuspended sample was sonicated for 5 minutes. The spheroplast fraction (pellet) and cytosolic fraction (supernatant) were collected separately by centrifugation at 10,000*g* for 20 minutes.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. Crude, as well as purified fusion proteins were analyzed on SDS-PAGE. SDS-PAGE was performed using 4% to 20% gradient gels (Bio-Rad, Richmond, CA) and a Mini-Protein II gel apparatus (Bio-Rad). Proteins were stained with Coomassie brilliant blue. For immunoblotting, electrophoresed proteins were transferred to nitrocellulose membranes. Membranes were blocked with 3% gelatin-containing TBS (20 mmol/L Tris, 500 mmol/L NaCl, pH 7.5) and washed with TTBS (TBS, 0.05% Tween-20, pH 7.5). Horse anti-DT sera (Connaught Lab, Switwater, PA) and anti–mIL-3 monoclonal antibody (MoAb; rat IgG_1; Genzyme, Cambridge, MA) were used as a source of primary antibodies. The blots were processed using horseradish peroxidase-conjugated protein-G (Protein G-HRP) and developed using HRP color reagents (Bio-Rad).

Isolation of inclusion bodies and renaturation and purification of the fusion proteins. The method of isolating the inclusion bodies

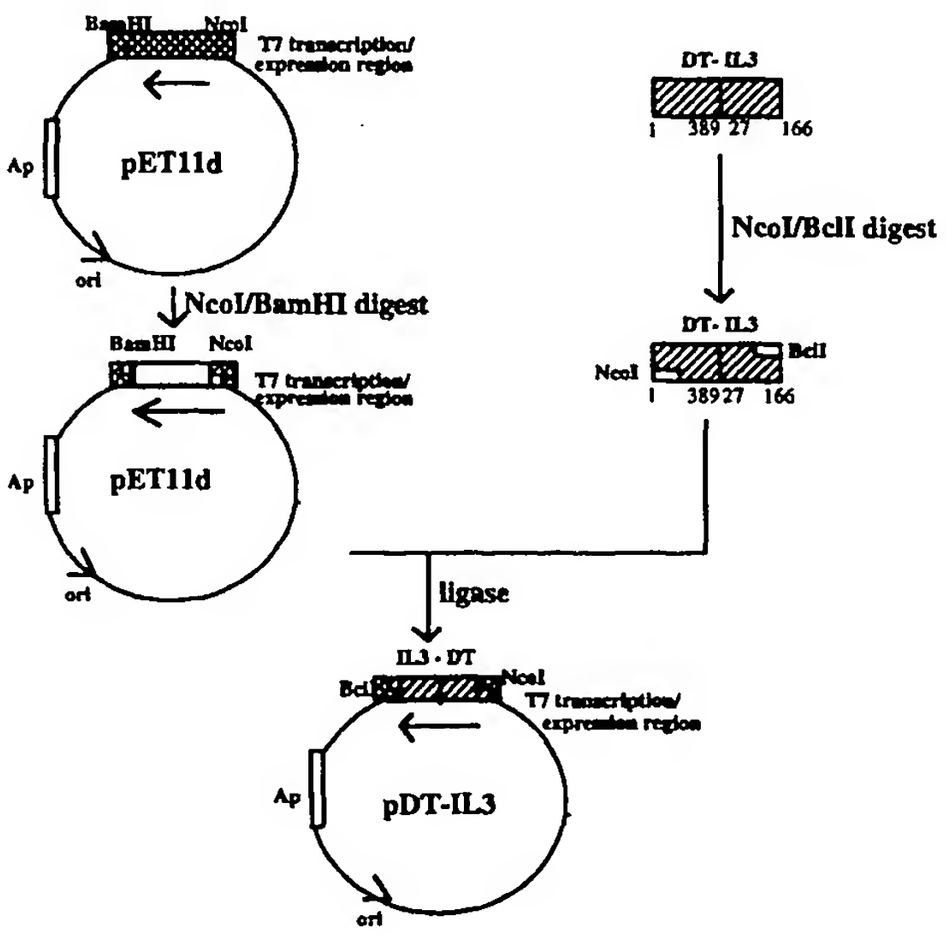

Fig 1. Assembly of the DT_{390}-mIL-3 gene in the pET11d vector. The hybrid gene encoding DT_{390}-mIL-3 was constructed by the method of gene splicing by overlap extension. The DT_{390}-mIL-3 hybrid gene was digested with restriction enzyme *Nco* I and *Bcl* I and ligated into the *Nco* I and *Bam*HI compatible cloning sites of a pET11d plasmid under the control of a T7 promoter.

was previously described.[33] In brief, bacterial pellets were resuspended in TE buffer (50 mmol/L Tris, pH 8.0, 20 mmol/L EDTA, 100 mmol/L NaCl) and treated with 0.02% lysozyme for 30 minutes. The pellet was then incubated in Triton X-100 buffer (11% vol/vol Triton X-100, 89% vol/vol TE) for 30 minutes at room temperature after briefly homogenizing with a tissuemizer (Thomas Scientifics, Germany). The pellets were washed 3 times with Triton X-100 buffer and 4 times with TE buffer by briefly homogenizing with a tissuemizer and incubating for 5 to 10 minutes. Inclusion bodies were collected by centrifugation at 24,000*g* for 50 minutes. Solubilization of the inclusion body pellet was achieved by sonicating in denaturant buffer consisting of 7 mol/L guanidine, 0.1 mol/L Tris, pH 8.0, and 2 mmol/L EDTA. Protein concentrations were determined by the Bradford method[35] and adjusted to 10 mg/mL with solubilization buffer. The solution was incubated at room temperature for 16 hours in the presence of 65 mmol/L dithioerythritol (DTE).

To remove insoluble material, the solution was centrifuged at 40,000*g* for 10 minutes and the supernatant was collected. Renaturation was initiated by a rapid 100-fold dilution of the denatured and reduced protein into chilled refolding buffer consisting of 0.1mol/L Tris, pH 8.0, 0.5 mol/L L-arginine, 0.9 mmol/L oxidized glutathione (GSSG), and 2 mmol/L EDTA. The samples were incubated at 10°C for 48 hours. The refolded protein was diafiltrated and ultrafiltrated against 20 mmol/L Tris, pH 7.8, using a spiral membrane ultrafiltration cartridge on Amicon's CH2 system (Amicon, Beverly, MA). Samples were loaded on a Q-Sepharose (Sigma) column and eluted with 0.3 mol/L NaCl in 20 mmol/L Tris, pH 7.8. The protein was diluted fivefold and subsequently applied to a Resource Q column (Pharmacia, Uppsala, Sweden) and eluted with a linear salt gradient from 0 to 0.4 mol/L NaCl in 20 mmol/L Tris, pH 7.8. The main peak from the Resource Q column was purified by size-exclusion chromatography on a TSK 250 column (TosoHass, Philadelphia, PA).

ADP ribosylation assay. Duplicate samples of nicked DT and DT_{390}-mIL-3 were examined for their ADP ribosyl transferase activity, as previously described.[33,36] The toxin was nicked by treating 15 μg of DT_{390}-mIL-3 with 0.04 μg of trypsin for 15 minutes at 37°C and the reaction was stopped with soybean trypsin inhibitor (Sigma). Briefly, ADP-ribosylation was performed in 80-μL reaction mixtures containing 40 μL of 0.01 mol/L Tris-HCl buffer with 1.0 mmol/L dithiothreitol, pH 8.0, 10 μL of rabbit reticulocyte lysate (containing elongation factor-2 [EF-2]), 10 μL 0.1% bovine serum albumin (BSA), and 10 μL of toxin sample. The reaction was initiated by the addition of 10 μL of 0.57 mmol/L [^{32}P] nicotinamide adenine dinucleotide (ICN Biomedicals, Irvine, CA). Reaction mixtures were incubated at room temperature for 1 hour and the reaction was stopped by the addition of 1 mL 10% trichloroacetic acid (TCA). The precipitate was collected by centrifugation and washed with 1 mL 10% TCA. The radioactivity was counted by standard scintillation techniques.

Cytotoxicity assay. To characterize the cytotoxic activity of DT_{390}-mIL-3, we used the murine myelomonocytic cell line FDC-P1[37] (provided by Immunex, Inc, Seattle, WA), which is dependent on mIL-3 for proliferation. Cultured FDC-P1 cells were maintained in complete culture media consisting of RPMI-1640 supplemented with 10% fetal bovine serum, 1% sodium pyruvate, 1% L-glutamine, 1% penicillin/streptomycin, and 10% WEHI-3B conditioned media.[38] We assayed the cytotoxic activity by measuring the ability of DT_{390}-mIL-3 to inhibit the proliferation of FDC-P1 cells. Cells were initially washed three times with nonsupplemented RPMI-1640 to remove any exogenous cytokine, followed by 1 hour of incubation at 37°C. Cells were seeded at 9×10^4 cells/tube in complete culture media and treated with one of the following toxins at concentrations ranging from 1×10^{-13} mol/L to 1×10^{-8} mol/L: DT_{390}-mIL-3, DT_{390}-hIL-2, DT_{390}-mIL-4, and native DT. The cells were treated for 4 hours at 37°C in a 5% CO_2 atmosphere.

After incubation, the cells were washed three times and then seeded at 3×10^4 cells/well in 96-well flat-bottomed plates in a volume of 200 μL. [H^3]-thymidine (1 μCi) and exogenous mIL-3 at a final concentration of 5 ng/mL were added into each well. After 24 hours, the cells were harvested on glass fiber filters and counted according to standard methods. Cells cultured with media alone served as the control. All assays were performed in triplicate. For kinetic analysis, cells were treated with DT_{390}-mIL-3, as described above, except that the designated toxin treatment intervals varied from 30 minutes to 8 hours. Three additional murine cell lines were used that did not respond to mIL-3: FDCP2.1d (a myelomonocytic cell line derived from FDCP2),[33] EL4 (a T-cell leukemia/lymphoma), and C1498 (a myeloid leukemia; American Type Culture Collection, Rockville, MD).

Granulocyte-macrophage colony-forming assay (CFU-GM). DT_{390}-mIL-3, DT_{390}-hIL2, or native DT was examined for its effects on committed BM progenitor cells in a CFU-GM assay, which was performed by culture of treated murine BM cells in complete methylcellulose medium (30% fetal calf serum, 1% pokeweed mitogen [PWM]-stimulated murine spleen cell-conditioned medium, 1% BSA, 0.9% methylcellulose, 10^{-4} mol/L 2-mercaptoethanol, and 3 U/mL erythropoietin; StemCell Technologies, Vancouver, British Columbia, Canada). Our CFU-GM-assay was previously described.[33] Briefly, BM cells were collected by flushing the shafts of femora and tibiae of C57BL/6 mice. Cells were resuspended at 5×10^4 cells/mL in complete methylcellulose medium and were plated in culture dishes for 14 days. Under an inverted microscope, colonies of greater than 50 cells were scored as CFU-GM according to their morphology.

During the kinetic studies, BM cells prepared as mentioned above were cultured with DT_{390}-mIL-3 at a final fusion toxin concentration of 10 nmol/L. After 4, 8, 12, or 16 hours, the treated cells were recovered by centrifugation for 10 minutes at 300*g* and the supernatant was decanted. The cells were washed with medium three times, resuspended at 5×10^4 cells/mL in complete methylcellulose medium, and scored as mentioned above.

Splenic colony-forming assay (CFU-S). Our CFU-S assay was previously described.[39] C57BL/6 BM cells were isolated and treated as described above. Treated BM cells (10^5) were injected into the lateral tail vein of sublethally irradiated (7.5 Gy Cesium at a dose rate of 57.72 rads/min; JL Shepherd and Associates, Glendale, CA) syngeneic mice. Eight or 13 days later, the spleens were removed and fixed with Bouins solution. Visible surface colonies were counted and scored as CFU-S. Control mice that received irradiation but no BM showed no macroscopic spleen colonies on day 8 or 13. Mice were housed in our AAALAC-accredited facility under specific pathogen-free (SPF) conditions in microbarrier cages.

Adoptive transfer studies. An adoptive transfer assay to rescue irradiated mice was modified from a previously described assay.[40] C57BL/6 Ly5.1 (10 mice/group) recipients were irradiated with lethal total body irradiation (9 Gy x-ray at a dose rate of 39.30 rads/min; Phillips Medical System, Brookfield, WI) 18 hours before intravenous injection with varying cell doses of DT_{390}-mIL-3–treated donor BM. Donor BM from C57BL/6 Ly5.2 congenic mice was treated with fusion toxin for 8 hours and then infused. Ly5.2 is an alleleic form of the Ly5.1 antigen expressed on all hematopoietic cells. These markers are typable by flow cytometry with fluorochrome-labeled MoAb, as we previously reported (staining with fluorescein isothiocyanate [FITC]–anti-Ly5.2 for donor cells and phycoerythrin [PE]-anti-Ly5.1 for host cells).[41] Recipient survival was monitored daily. The irradiated mice were confirmed as rescued by donor-derived cells if they survived for 30 days. Animals died on days 5 through 10 in these radiation protection studies. It is possible

that these deaths were related to the presence of bacteria in our SPF colony requiring larger doses of BM to effect radioprotection. However, attempts to isolate Proteus vulgaris, Pseudomonas species, or Klebsiella species from the colon, oropharynx, or ileum of sentinel mice were unsuccessful.

Competitive repopulation studies. The competitive repopulation assay was performed as previously described, with some modification.[32] C57BL/6 Ly5.1 recipient mice (10 mice/group) were lethally irradiated (9 Gy). Eighteen hours later, mice received a mixture of C57BL/6 Ly5.1 and congenic C57BL/6 Ly5.2 BM cells. The C57BL/6 Ly5.2 BM cells were preincubated with either DT_{390}-mIL-3 or medium alone and the C57BL/6 Ly5.1 BM cells were preincubated with medium. After 8 hours of treatment, BM cells were thoroughly washed with media 3 times to remove the fusion toxin. Equal numbers of Ly5.1 (2.5×10^6) and Ly5.2 (2.5×10^6) cells were mixed together and injected intravenously into irradiated Ly5.1 mice. The percentage of Ly5.2 and Ly5.1 cells in the peripheral blood, bone marrow, spleen, and thymus were determined by flow cytometry by staining with FITC–anti-Ly5.2 and PE–anti-Ly5.1 MoAb as described above.

Statistical analysis. Group comparisons of data in Tables 2 through 4 were made using the Student's *t*-test. *P* values $\leq .05$ were considered significant.

RESULTS

Genetic construction of DT_{390}-mIL-3. The DNA fragments encoding the structural gene for DT_{390} and mIL-3 were obtained by separate PCRs with the sizes of 1,197 bp and 467 bp, respectively. After the third PCR, the resulting SOE product, DT_{390}-mIL-3 hybrid gene, was generated with a 1,631-bp size. The DT_{390}-mIL-3 hybrid gene encodes an *Nco* I restriction site, an ATG initiation codon, the first 389 amino acids of the DT, the mature murine IL-3 polypeptide, six histidine tag, a TAA stop codon, and a *Bcl* I compatible restriction site. After digestion, the DT_{390}-mIL-3 hybrid gene was cloned into the pET11d plasmid under the control of the IPTG inducible T7 promoter to create a 7,255-bp pDT-IL-3 plasmid (Fig 1). Restriction endonuclease digestion verified that the DT_{390}-mIL-3 hybrid gene sequence had been cloned in frame. DNA sequencing analysis was performed by the University of Minnesota Microchemical Facilities (University of Minnesota, Minneapolis, MN) and no mutations were detected.

Expression and purification of DT_{390}-mIL-3 fusion protein. Expression of the fusion protein in *E coli* was induced with IPTG at either 30°C or 37°C with the same efficiency (data not shown). Coomassie brilliant blue-stained SDS-polyacrylamide gel of whole bacterial lysate after IPTG induction showed a novel expressed protein migrating at 58 kD, which corresponds to the expected size for DT_{390}-mIL-3 protein (Fig 2, lane 3). The localization study of the expressed fusion protein showed that DT_{390}-mIL-3 was retained in the inclusion bodies (Fig 2, lanes 4 through 6). To extract the DT_{390}-mIL-3 protein, the inclusion bodies were isolated and denatured, and the protein was refolded as described in the Materials and Methods. After the renaturation procedure, the crude DT_{390}-mIL-3 was purified by sequential chromatography. The elution from the anion-exchange Q-sepharose and resource Q columns showed an enrichment of a protein with an electrophoretic mobility corresponding to an apparent molecular mass of 58 kD (Fig 2, lane 7). To further purify

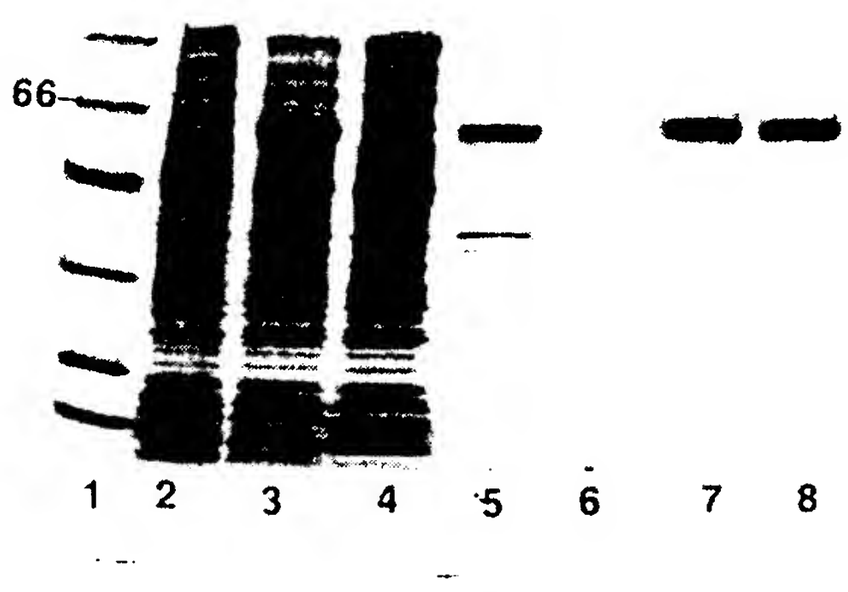

Fig 2. SDS-PAGE of the purified DT_{390}-mIL-3 protein stained with Coomassie blue. Lane 1, molecular weight standard; lane 2, uninduced total bacteria lysate; lane 3, IPTG-induced total bacteria lysate; lane 4, fraction of soluble protein in cytoplasm; lane 5, fraction of insoluble protein in inclusion body; lane 6, soluble protein in periplasmic space; lane 7, major eluate from anion-exchange column; and lane 8, major peak from HPLC sizing column. The molecular weight standards are at 97, 66, 45, 31, 22, and 14 kD.

this fusion protein, pooled peak fractions from the anion-exchange columns were subjected to high-performance liquid chromatography (HPLC) using a TSK-250 sizing column. The final product was greater than 95% pure (Fig 2, lane 8). The endotoxin level of the purified DT_{390}-mIL-3 was less than 2 EU/mg protein as detected by limulus amebocyte lysate (LAL) assay. Additional analysis of this DT_{390}-mIL-3 protein by immunoblotting was performed. Anti-DT sera and anti–mIL-3 MoAbs were able to recognize the renatured and purified DT_{390}-mIL-3. The control native DT and DT_{390}-hIL-2 were immunoblotted with the anti-DT sera but not the anti–mIL-3 MoAb.

Enzymatic activity and in vitro cytotoxicity. Protein synthesis inhibition by DT is due to fragment A-catalyzed ADP-ribosylation of cytoplasmic EF-2. To determine whether the DT_{390}-mIL-3 protein also displays such enzymatic activity, a cell-free assay system was used in which rabbit reticulocyte lysate, a source of EF-2, was exposed to either native DT or DT_{390}-mIL-3 in the presence of [^{32}P]-NAD. Incubation with either native DT or DT_{390}-mIL-3 toxin showed a similar dose-dependent increase in [^{32}P] incorporation into the TCA-precipitable fraction of rabbit reticulocyte lysate (EF-2). In contrast, the negative control of BSA did not show such an ADP-ribosylation activity. This result confirmed that DT_{390}-mIL-3 possessed ADP-ribosyl transferase activity.

To characterize the cytotoxic activity of DT_{390}-mIL-3, a bioassay was devised using the mIL-3–dependent myelomonocytic leukemia cell line FDC-P1 that expresses IL-3R and is readily stimulated by IL-3. In our hands, IL-3–induced stimulation of FDC-P1 cells was dose-dependent and 16 ng/mL IL-3 resulted in a curve plateau with 350,000 cpm in a thymidine uptake assay representing a 100-fold increase compared with background. The cytotoxicity was evaluated by measuring the inhibition of cellular proliferation. The ability of various concentrations of DT_{390}-mIL-3 to inhibit

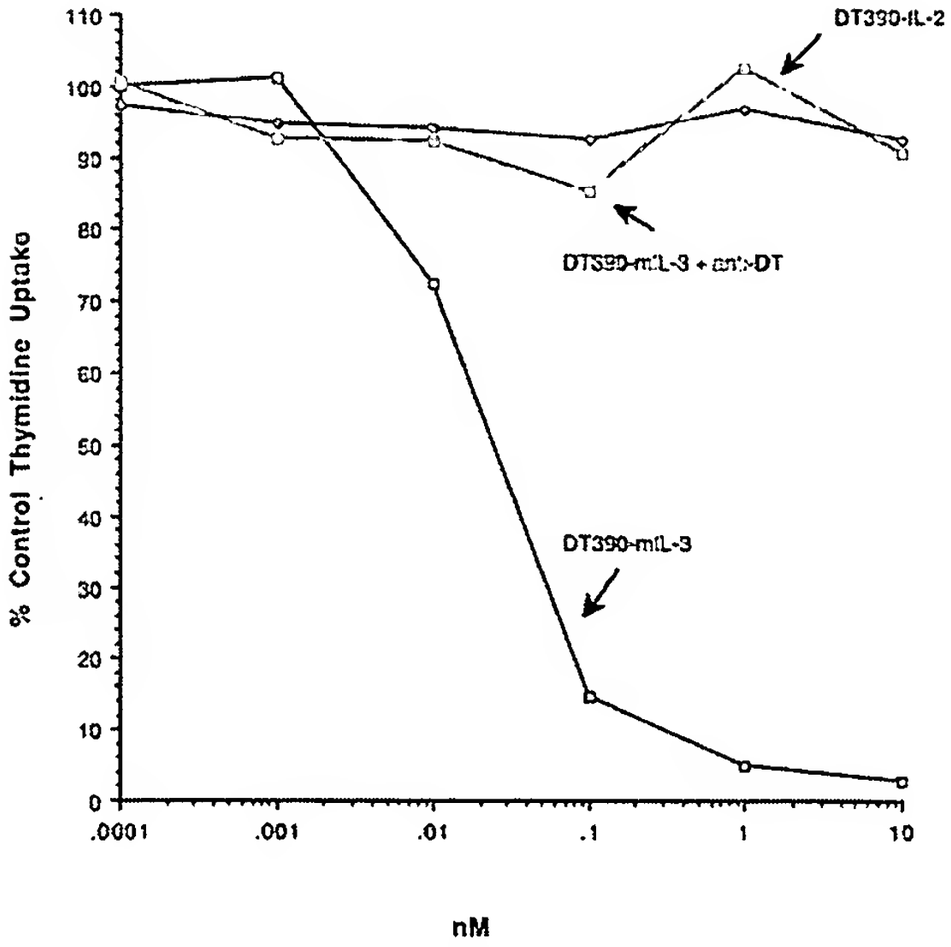

Fig 3. Cytotoxic activity of fusion toxins on FDC-P1 cells. Various amounts of DT_{390}-mIL-3 or DT_{390}-hIL-2 were added to FDC-P1 cells for 4 hours. After washing, the percentage of incorporation of [H^3] thymidine relative to controls not treated with fusion toxin was determined. Neutralization of DT_{390}-mIL-3 was studied by adding 0.1% anti-DT antiserum during the 4 hours of incubation.

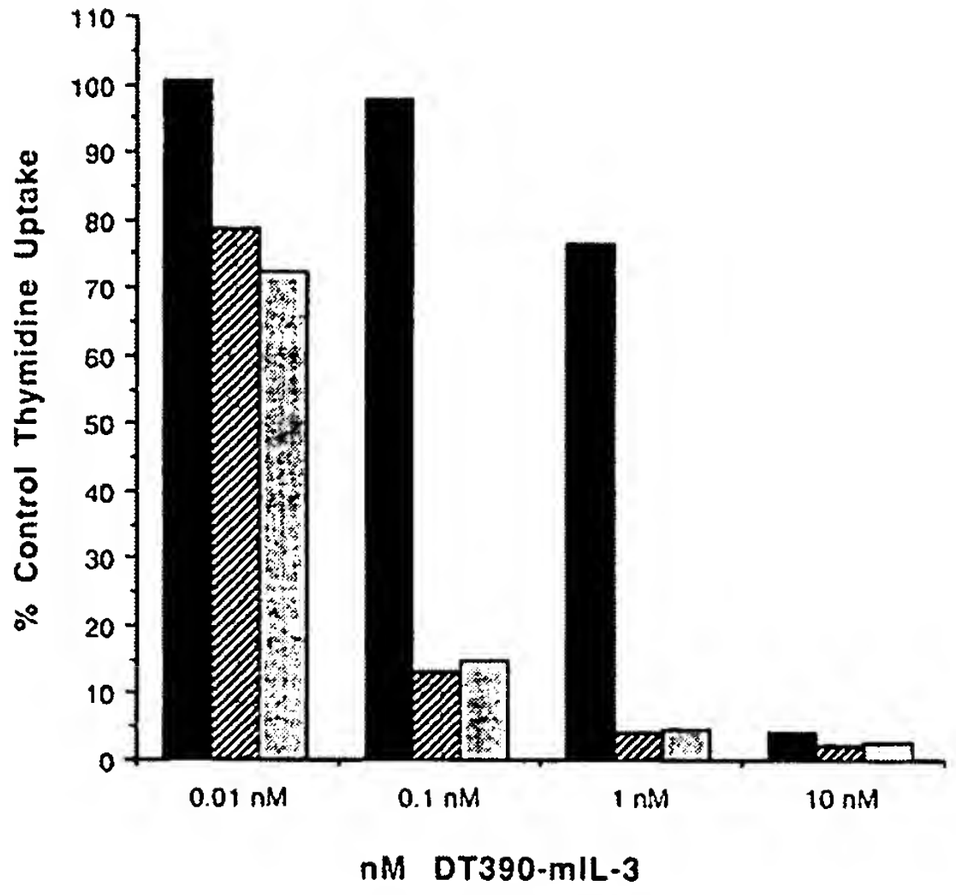

Fig 4. Neutralization of DT_{390}-mIL-3 by anti-mIL-3 and irrelevant antibody. Various concentrations of DT_{390}-mIL-3 were incubated with (■) 10 nmol/L of anti-mIL-3 antibody or (▨) irrelevant anti-Thy1.2 antibody and were added to FDC-P1 cells as described in Fig 3. (□) DT_{390}-mIL-3 alone. Results are expressed as the percentage of activity in control cultures not treated with fusion protein.

the proliferation on FDC-P1 cells was examined. FDC-P1 cells were inhibited by DT_{390}-mIL-3 in a dose-dependent manner with an IC_{50} of 0.025 nmol/L (or 1.5 ng/mL; Fig 3). To determine if the cytotoxic activity of DT_{390}-mIL-3 on FDC-P1 cells was mediated by the binding of the mIL-3 moiety, FDC-P1 cells were cultured with DT_{390}-hIL-2, an irrelevant fusion toxin control. IL-2 did not stimulate the growth of this cell line; thus, the IL-2R is not expressed on FDC-P1 cells (data not shown). In contrast to DT_{390}-mIL-3, FDC-P1 cells were resistant to as much as 10 nmol/L DT_{390}-hIL-2. The cytotoxic effect of DT_{390}-mIL-3 was fully neutralized by 0.1% anti-DT sera (Fig 3), which suggested that the cytotoxicity of DT_{390}-mIL-3 was mediated by the enzymatic activity of DT_{390} fragment. Furthermore, anti-mIL-3 antibodies blocked the cytotoxic effect of DT_{390}-mIL-3 (Fig 4). The addition of 10 nmol/L anti-mIL-3 antibodies fully neutralized the cytotoxic effect of 0.01 nmol/L or 0.1 nmol/L DT_{390}-mIL-3 and neutralized the cytotoxic effect of 1 nmol/L DT_{390}-mIL-3 up to 80%. However, 10 nmol/L of anti-mIL-3 antibodies did not block the cytotoxic effect of 10 nmol/L DT_{390}-mIL-3. The irrelevant anti-Thy1.2 antibodies did not block the activity of DT_{390}-mIL-3. IL-3 was not used to block the cytotoxic activity of DT_{390}-mIL-3 in this study because IL-3 itself was used to stimulate the proliferation of FDC-P1 cells.

The effect of DT_{390}-mIL-3 on kinetics of proliferative inhibition to FDC-P1 cells was plotted as a percentage of the control level versus a function of time (Fig 5). As expected, the rate of inhibition was concentration-dependent, with higher concentrations giving faster rates. The kinetics were rapid because a treatment as short as 30 minutes resulted in 90% inhibition. After 8 hours, 98% inhibition was observed. Cellular proliferative inhibition increased linearly for those toxin concentrations of either 1 nmol/L or 0.1 nmol/L. These kinetics indicate a first-order single-hit process.

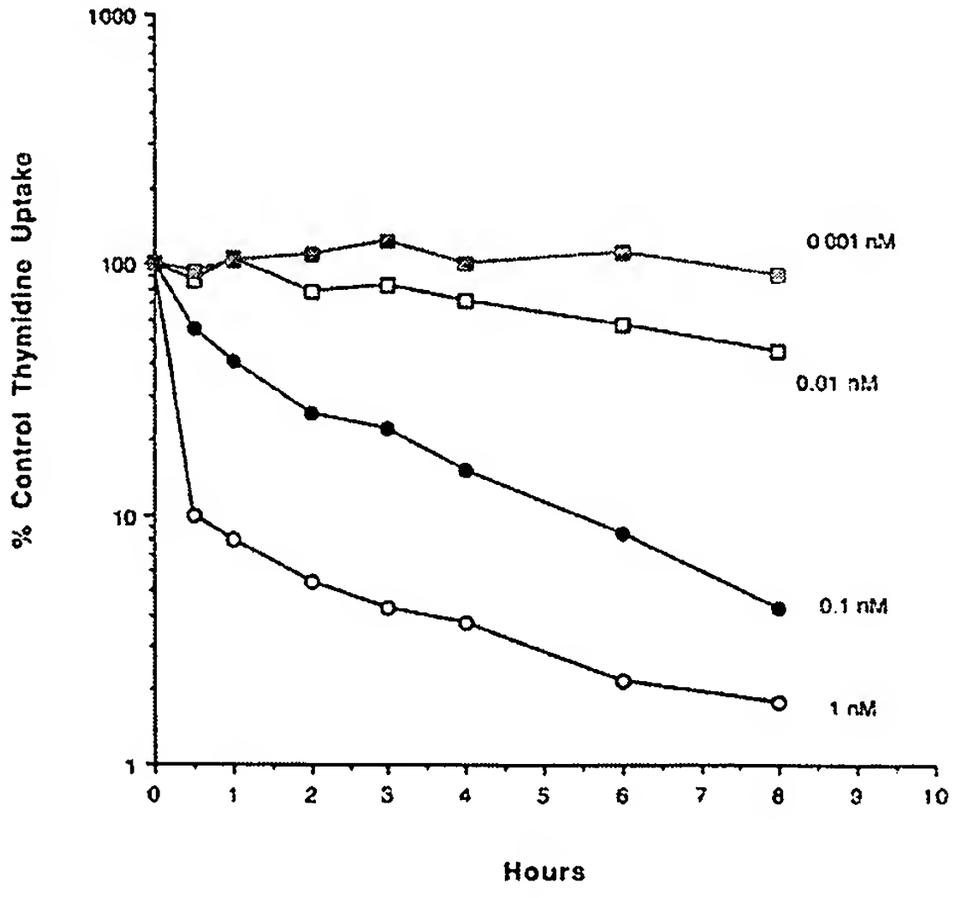

Fig 5. For kinetic analysis, FDCP.1 cells were treated with DT_{390}-mIL-3 as described in Fig 3, except that the designated toxin treatment intervals varied from 30 minutes to 8 hours. The effect of DT_{390}-mIL-3 on kinetics of proliferative inhibition to FDCP.1 cells was plotted as a percentage of the control level versus a function of time.

DT_{390}-mIL-3 had no effect on cell lines that did not express IL-3R. The FDC-P2.1d cell line, which was also derived from the FDC-P parental cell line, is murine granulocyte-macrophage colony-stimulating factor (mGM-CSF)–dependent and mIL-3 nonresponsive. It was not inhibited by DT_{390}-mIL-3 (data not shown). Furthermore, DT_{390}-mIL-3 showed a high IC_{50} of greater than 2×10^{-8} mol/L on the T-cell line EL4 and the myelomonocytic leukemia cell line C1498 (data not shown). Together, these reduced effects on IL-3 nonresponsive lines indicate that DT_{390}-mIL-3 was specifically cytotoxic to cells via the IL-3 ligand-receptor complex.

The effect of DT_{390}-mIL-3 on hematopoietic progenitor cells. It has been well documented that IL-3 plays a role in the development of multiple lineages in hematopoiesis.[1,2] It is of interest to know at which stages of development hematopoieic progenitor cells express the high-affinity IL-3R. To determine the effect of DT_{390}-mIL-3 on committed myeloid progenitor cells, we tested its ability to inhibit CFU-GM colonies. DT_{390}-mIL-3 inhibited colony formation 95% and 99% at toxin concentrations of 1 nmol/L or 10 nmol/L, respectively (Fig 6A). In contrast, DT_{390}-hIL-2, an irrelevant control fusion toxin, had little inhibitory effect. Native DT also had little effect. This is consistent with previous reports that mouse cells are more resistant to DT because they lack DT receptors.[43,44] Kinetic studies (Fig 6B) showed a relationship between the increasing inhibition of CFU-GM and incubation time. Approximately 2 log inhibition was obtained after 8 hours. We chose this 8-hour incubation time for subsequent studies because untreated, cultured BM underwent a 40% reduction in CFU-GM colony number at longer incubation intervals. Under these culture conditions, 70% CFU-GM colonies were still preserved in untreated BM after 8 hours of culture. Concentrations of fusion toxin greater than 10 nmol/L resulted in nonspecific killing.

To test the effect of DT_{390}-mIL-3 on progenitor cells earlier than those giving rise to CFU-GM colonies, CFU-S assays were performed. CFU-S are heterogeneous with respect to their proliferative capacity, self-renewal ability, and cell cycle status.[45,46] The progenitors that produce CFU-S colonies represent earlier committed progenitor cells, but not primitive progenitor cells. Day-8 CFU-S have little self-renewal potential and represent committed hematopoietic progenitors more closely related to erythroid progenitors (burst-forming unit-erythroid [BFU-E]) or granulocyte-macrophage progenitors (CFU-GM). Day-13 CFU-S contain cells with more than one lineage.[47,48] However, recent evidence suggests that neither day-8 CFU-S nor day-13 CFU-S represent the pluripotent hematopoietic stem cells.[49-51] DT_{390}-mIL-3 was used at a dose of 10 nmol/L in an 8-hour preincubation because we knew that higher doses would result in nonspecific killing of CFU-S. At this dose, the DT_{390}-mIL-3 inhibited day-8 CFU-S about 80% and day-13 CFU-S about 50% (Fig 7). The inhibition of CFU-S colonies was specific and mediated by the DT portion of the molecule because the anti-DT sera was able to neutralize the CFU-S inhibition.

Together, the combined CFU-GM and CFU-S studies indicate that the high-affinity IL-3R are expressed on committed BM progenitor cells and that the progenitor cells of day-8 CFU-S are more sensitive to DT_{390}-mIL-3 treatment than those of day-13 CFU-S.

The ability of DT_{390}-mIL-3–treated cells to rescue lethally irradiated mice after adoptive transfer. To determine the effect of DT_{390}-mIL-3 on the progenitor cells capable of providing radiation protection, adoptive transfer assays were performed. C57BL/6 Ly5.2 BM cells were treated with 10 nmol/L DT_{390}-mIL-3. After washing, 1, 2, or 4×10^5 treated cells were injected into lethally irradiated (9.0Gy) C57BL/6 Ly5.1 congenic recipients. The results showed that all of these recipients survived less than 19 days. When a higher dose (8×10^6) of DT_{390}-mIL-3 treated cells was injected, 60% of the recipients survived more than 30 days, indicating that recipients could be rescued with high BM doses despite

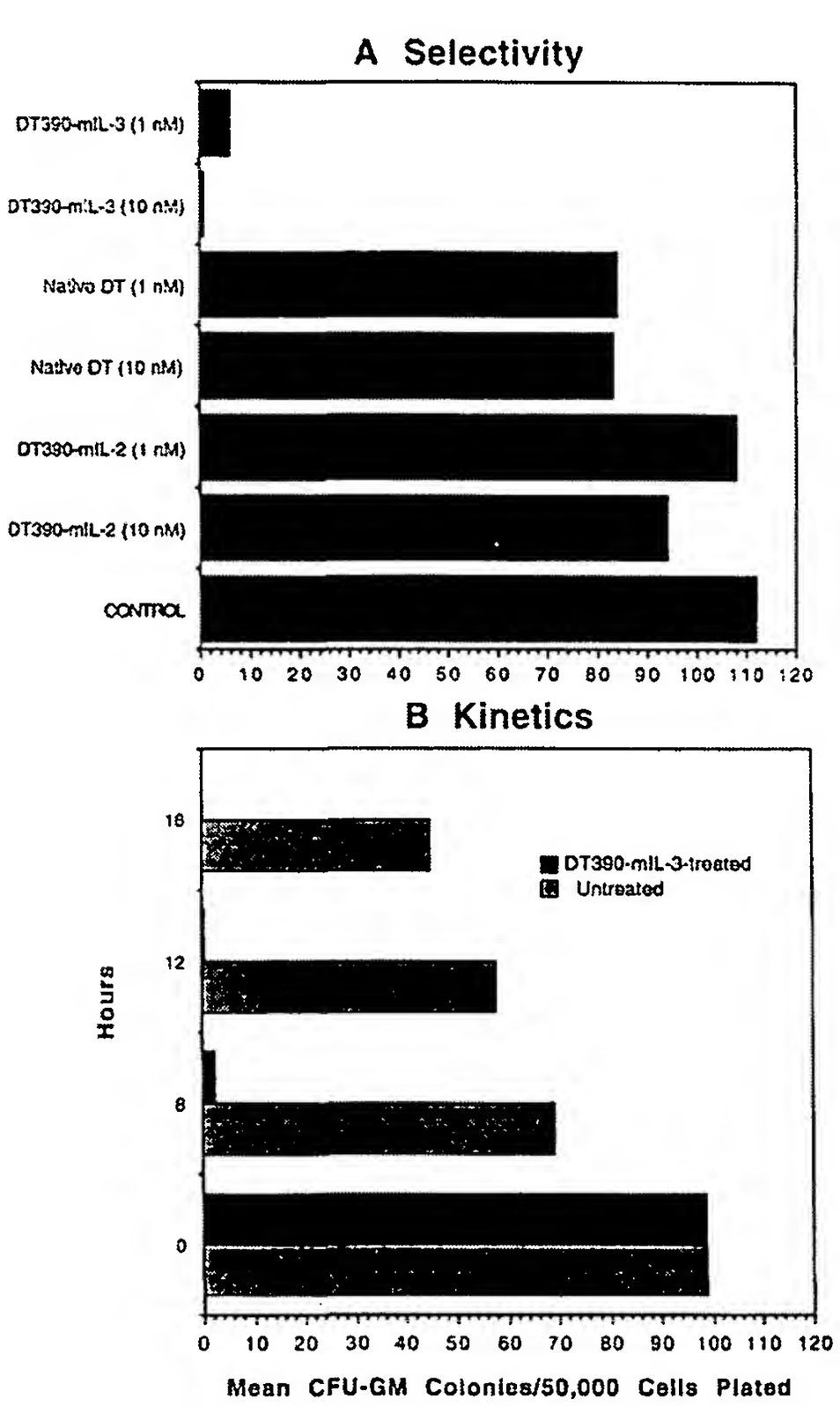

Fig 6. (A) Effect of DT_{390}-mIL-3 on the colony formation of CFU-GM myeloid progenitor cells. Mouse BM cells were incubated with either 1 nmol/L or 10 nmol/L fusion toxin for 8 hours. Toxin was removed by washing BM cells 3 times. Colony number was determined relative to 5×10^4 mononuclear BM cells plated on semisolid methylcellulose media in an 1-mL culture volume. (B) Mouse BM cells were treated with 10 nmol/L DT_{390}-mIL-3 or media alone for 8, 12, or 16 hours. After washing, the CFU-GM was assayed as in (A).

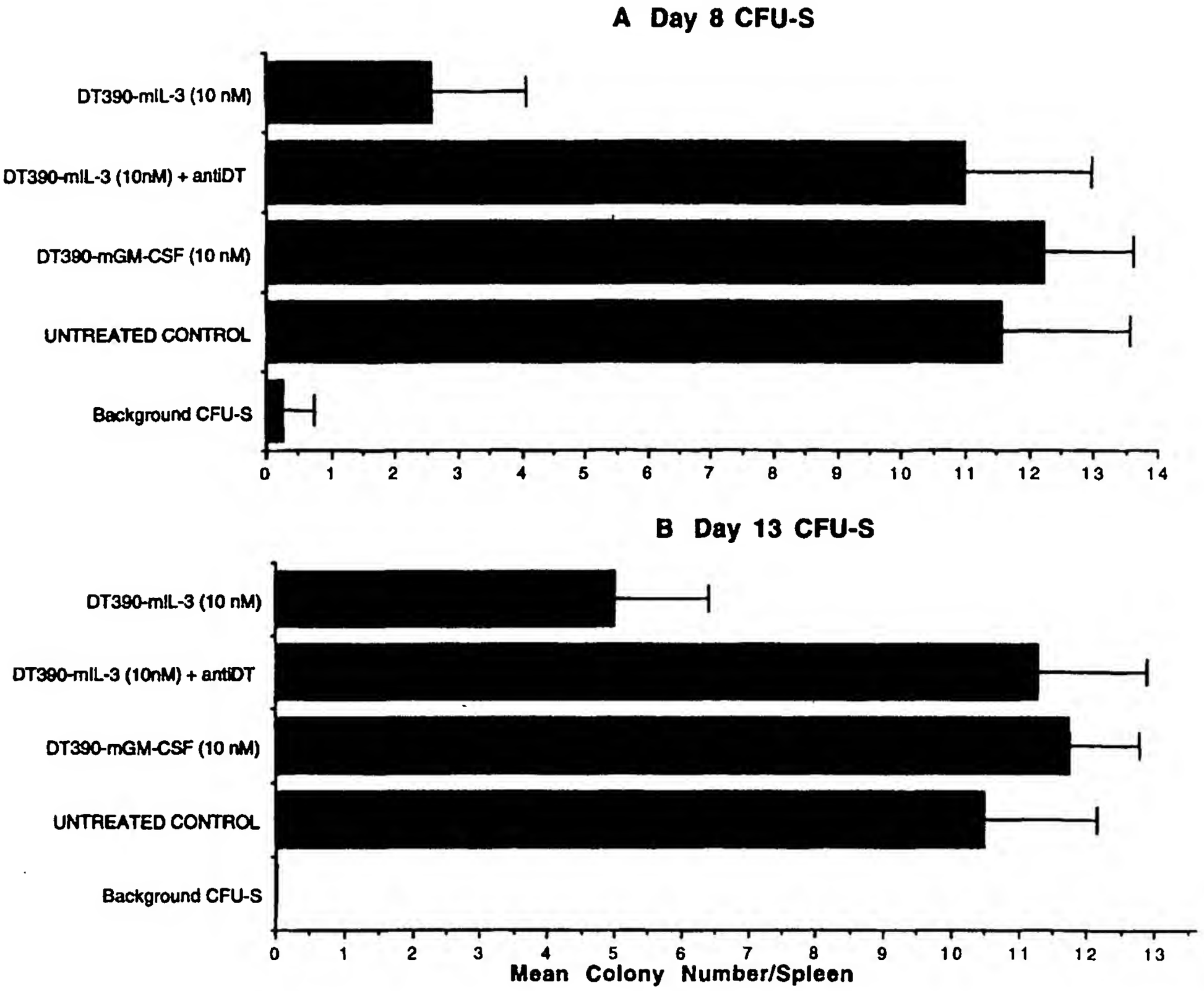

Fig 7. CFU-S activity on day 8 and day 13 of fusion toxin-treated BM cells. BM cells were incubated with toxin for 8 hours. After washing, BM cells (1×10^5) were injected into syngeneic irradiated (7.5 Gy Cs) recipients (C57BL/6). Mice were killed either 8 or 13 days later. The spleens were removed and fixed in Bouins solution. Visible surface colonies were counted and scored (n = 7 to 8 mice/group).

treatment. Sixty percent of recipients survived when 1×10^6 nontreated cells were administered, indicating that DT_{390}-mIL-3 treatment removed about 87.5% of the progenitor cells capable of rescue. All recipients survived more than 30 days when the administered cell doses of nontreated cells were greater than 2×10^6. Earlier published studies showed that DT_{390}-mGM-CSF killed committed myeloid progenitors[33] and Fig 8 showed that 2×10^6 DT_{390}-mGM-CSF–treated cells rescued 60% of the mice. A comparison of this curve to the curve in which mice received nontreated BM indicated that, with DT_{390}-mGM-CSF treatment, there was a removal of about 50% of the progenitor cells capable of rescue. All the recipients that survived for the first 30 days survived until the termination of the experiment on day 110.

On day 30, we typed donor peripheral blood leukocytes and confirmed that the survivors were indeed reconstituted with donor-type cells. The results given above suggested that DT_{390}-mIL-3 kills more progenitor cells capable of BM rescue than does DT_{390}-mGM-CSF.

The ability of DT_{390}-mIL-3–treated cells to competitively repopulate irradiated mice in BM mixing experiments. To determine whether DT_{390}-mIL-3 was acting on more primitive BM progenitor cells capable of long-term repopulation, competitive repopulation studies were performed. When differing proportions (percentages) of C57BL/6 and congenic C57BL/6 Ly5.2 BM cells totalling 10 million were mixed (100/0, 83/17, 50/50,17/83, and 0/100) and transplanted into groups (n = 5/group) of irradiated syngeneic recipients, similar proportions of reconstituting cells were measured in the peripheral blood by flow cytometry several weeks later (100/0, 84/16, 53/47, 13/87, and 6/94). This finding indicated that reconstituting cells correlated with the original proportion of BM progenitor cells and that a model was operational in which the effects of DT_{390}-mIL-3 could be determined.

In separate experiments, C57BL/6 Ly5.2 BM cells were treated with DT_{390}-mIL-3 and then mixed with an equal proportion of untreated C57BL/6 Ly5.1 BM cells. Mixed BM cells were injected into lethally irradiated (9.0 Gy) C57BL/

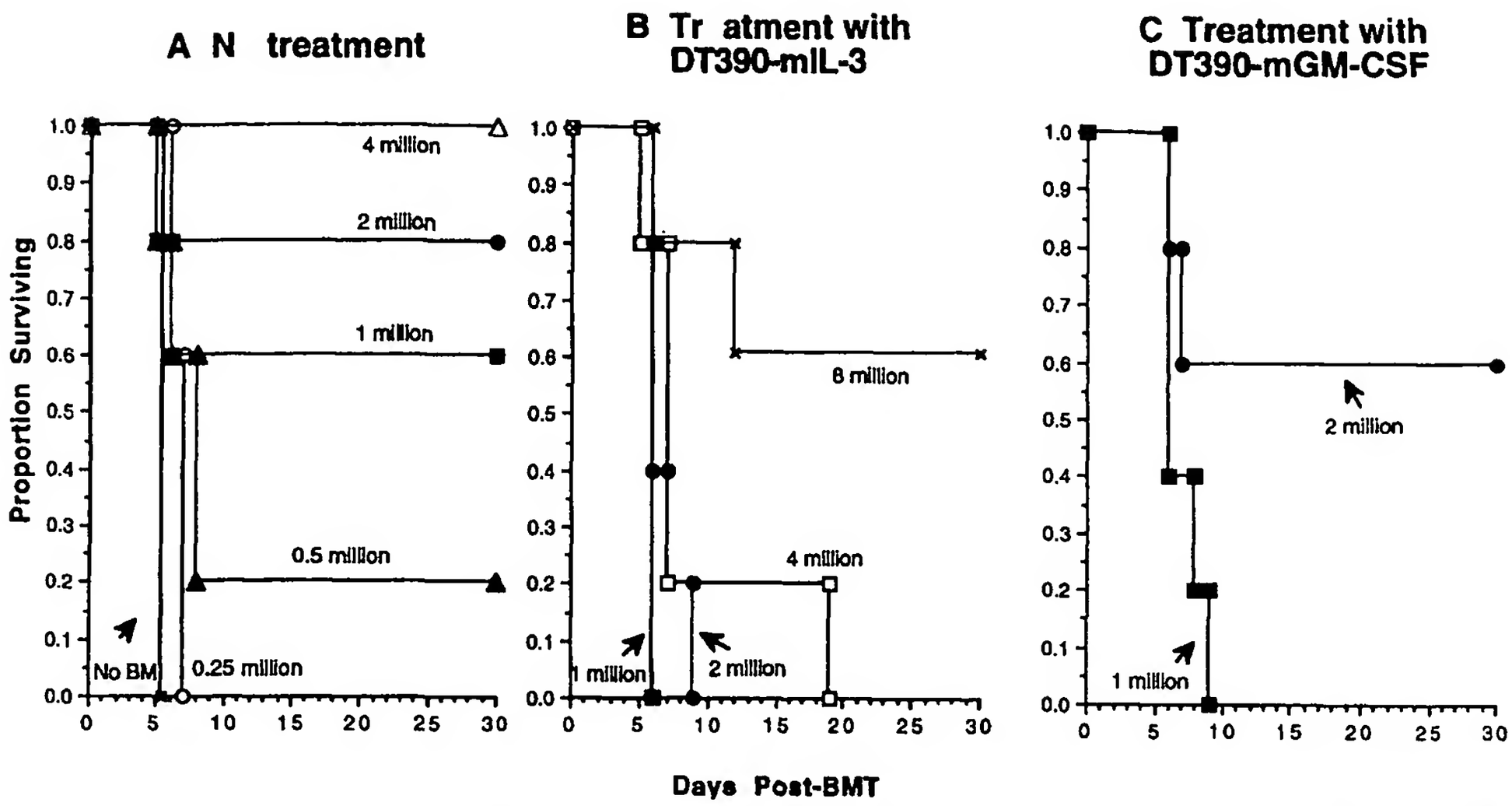

Fig 8. Adoptive transfer BM rescue experiments. C57BL/6 Ly5.2 congenic BM cells were incubated with or without DT_{390}-mIL-3 or DT_{390}-mGM-CSF for 8 hours. After washing, various doses of BM cells were injected into irradiated (9 Gy) C57Bl/6 Ly5.1 recipients. Recipient survival was monitored daily.

6 Ly5.1 recipients. Peripheral blood cells of recipients were studied by flow cytometry for the presence of Ly5.1 and Ly5.2-expressing cells 28 to 31 days after transplantation. Table 2 shows that, in 2 experiments, the mice receiving the DT_{390}-mIL-3–treated Ly5.2 BM cells had less reconstitution of Ly5.2-expressing cells as compared with the mice receiving nontreated Ly5.2 BM cells. Long-term reconstitution was also affected. On day 95 post-BM transplantation (BMT), various organs from animals from experiment 1 were typed. DT_{390}-mIL-3 inhibited reconstitution of Ly5.2 cells in blood, spleen, and thymus (Table 3). Interestingly, the inhibition of Ly5.2 cells was not complete. A failure of the fusion toxin to inhibit Ly5.2 BM cells in the treated culture is not a likely explanation because CFU-GM measured in these same treated BM cells was inhibited 92% (data not shown). It is more likely that some primitive progenitors express enough high-affinity IL-3R to be intoxicated by DT_{390}-mIL-3, whereas others do not, or that there might be a difference in receptor number.

To prove that pluripotent hematopoietic stem cells were indeed present on day 95 post-BMT, BM cells from a group of these mice were adoptively transferred to irradiated recipients (Table 4). Upon secondary transfer, these mice reconstituted with both Ly5.1 and Ly5.2 BM cells. Levels of Ly5.2 BM cells were still significantly lower than levels of Ly5.1 BM cells in the mice that received cells from animals that had originally received DT_{390}-mIL-3–treated BM cells. Together, these findings indicate that DT_{390}-mIL-3 is reactive against some but not all of the primitive progenitor cells.

Table 2. Competitive Repopulation of the Blood With DT_{390}-mIL-3–Treated Ly5.2-Expressing Cells on Days 28 Through 31 Post-BMT

	Experiment 1		Experiment 2	
	Ly5.1	Ly5.2	Ly5.1	Ly5.2
Untreated	55 ± 5	45 ± 5	61 ± 8	39 ± 8
DT_{390}-mIL-3	62 ± 1*	38 ± 1*	81 ± 5*	19 ± 5*

C57BL/6 Ly5.2 BM cells were treated with 10^{-8} mol/L DT_{390}-mIL-3 for 8 hours. Treated cells were adjusted and mixed with C57BL/6 Ly5.1 congenic BM cells so that equal concentrations (2.5×10^6 of Ly5.2 and 2.5×10^6 of Ly5.1 BM cells) were administered to groups of lethally irradiated C57BL/6 Ly5.1 recipients. To determine competitive repopulation, 4 to 8 recipients were studied by flow cytometry on days 28 through 31 post-BMT for the presence of Ly5.1- or Ly5.2-expressing cells, as described. Data are expressed as a mean percentage of positive cells ± 1 standard error of the mean.

* $P < .04$ compared with untreated controls.

DISCUSSION

The unique contribution of this work is the construction and description of a fusion toxin, DT_{390}-mIL-3, by genetically splicing the DNA segment encoding the ADP-ribosyl transferase enzymatic and hydrophobic translocation enhancing region of DT, but not the native binding site, to the DNA segment encoding the amino acids of the mature mIL-3 molecule. The fusion toxin was potent and highly selective in its activity because we measured an IC_{50} (concentration inhibiting 50% activity) of about 0.025 nmol/L (or 1.5 ng/mL) against the myelomonocytic cell line FDC-P1. Although amply equipped to kill leukemia cells, DT_{390}-mIL-3 might pose an equal threat to nonmalignant hematopoietic progenitor cells. The synthesis of the mouse reagent permitted the

Table 3. Long-Term Competitive Repopulation of the Various Tissues With DT_{390}-mIL-3–Treated Ly5.2-Expressing Cells on Day 95 Post-BMT

	Blood		BM		Spleen		Thymus	
	Ly5.1	Ly5.2	Ly5.1	Ly5.2	Ly5.1	Ly5.2	Ly5.1	Ly5.2
Untreated	39 ± 3	61 ± 3	52 ± 5	48 ± 5	36 ± 3	64 ± 3	39 ± 7	61 ± 7
DT_{390}-mIL-3	56 ± 5*	44 ± 5*	61 ± 6	39 ± 6	55 ± 5*	45 ± 5*	62 ± 13*	38 ± 13*

C57BL/6 Ly5.2 BM cells were treated with 10^{-8} mol/L DT_{390}-mIL-3 for 8 hours. Treated cells were adjusted and mixed with C57BL/6 Ly5.1 congenic BM cells so that equal concentrations (2.5×10^6 of Ly5.2 and 2.5×10^6 of Ly5.1 BM cells) were administered to groups of lethally irradiated C57BL/6 Ly5.1 recipients. To determine competitive repopulation, 4 recipients were studied by flow cytometry on day 95 post-BMT for the presence of Ly5.1- or Ly5.2-expressing cells as described. Data are expressed as a mean percentage of positive cells ± 1 standard error of the mean. These same DT_{390}-mIL-3–treated C57BL/6 Ly5.2 BM cells were plated in in vitro colony assays and were found to have a 90% reduction in CFU-GM.

* $P < .04$ compared with untreated controls.

evaluation of its reactivity against primitive progenitor cells using adoptive transfer experiments and competitive repopulation experiments not possible in humans. These data indicate that IL-3R may not be expressed uniformly on primitive BM progenitor cells.

The expression of IL-3R on hematopoietic stem cells at early stages of development is still a highly controversial issue.[9-11] We approached this issue by testing the ability of a fusion toxin directed against the IL-3R to eliminate BM progenitor cells at various stages of commitment. The most committed progenitor cell that we evaluated was that which gave rise to CFU-GM colonies. First, a working treatment schedule was established based on thymidine incorporation and colony assays. DT_{390}-mIL-3 reproducibly inhibited about 99% CFU-GM colony growth, showing that IL-3R is expressed on most myeloid-committed cells. These findings agree with previous reports. For example, Ogata et al[10] and Sugiura et al[52] reported that the hematopoietic progenitors can be separated into two populations by MoAbs against IL-3R–associated antigen(s) (IL-3RAA). Early differentiating progenitors (day-8 CFU-S and CFU-GM) were enriched in the IL-3RAA$^+$ cell population, whereas more immature multipotential progenitors (day-12 to -14 CFU-S and CFU-GEMM) were contained in the IL-3RAA$^-$ cell population. Therefore, IL-3RAA$^+$ cells are more committed than IL-3RAA$^-$ cells.

CFU-S colonies are considered to be derived from more primitive progenitor cells than CFU-GM. Most day-8 CFU-S colonies are thought to be committed to the erythroid lineage and sensitive to 5-fluorouracil (5-FU), whereas day-12 to -14 CFU-S are thought to be more uncommitted with multilineage potential and be resistant to 5-FU.[45,46] We found that the more committed day-8 CFU-S was more susceptible to the inhibitory effects of DT_{390}-mIL-3 (80%) than day-13 CFU-S (50%). These findings (1) provide further evidence that IL-3R is expressed on committed hematopoietic progenitor cells; (2) indicate that IL-3R is expressed on cells as primitive as day-12 to -14 CFU-S; and (3) suggest that more IL-3R are present on progenitor cells in the cycling phase than the Go phase. In other words, day-12 to -14 CFU-S are heterogeneous in their expression of IL-3R.

It is noteworthy that only high-affinity IL-3R are internalized when bound to their ligands. Although, theoretically, one DT molecule into the cytoplasm of the cell is required to produce cell death, the process of internalization and translocation into the cytoplasm is relatively inefficient, with only about 10% of the molecules that bind to the surface receptors entering the cytoplasm.[53,54] Thus, it is reasonable to infer that a critical number of growth factor-toxin molecules must be able to bind to cell surface receptors to cause subsequent internalization and cell death and that the cells expressing larger numbers of receptors have a higher chance of being intoxicated by specific growth factor-toxin molecules. Thus, differential sensitivity to DT_{390}-mIL-3 treatment between fractions of progenitor cells may be derived from (1) different affinities of IL-3R (high-affinity *v* low-affinity); (2) different numbers of high-affinity IL-3R; and (3) heterogeneity in the expression of IL-3R (presence *v* absence of receptors on progenitors). In these studies, progenitor cell resistance to DT_{390}-mIL-3 treatment could be explained by the absence of high-affinity IL-3R or only a small number of high-affinity IL-3R.

The most primitive stem cells, termed pluripotent hematopoietic stem cells (PHSC), are defined operationally by their (1) ability to rescue animals from lethal irradiation, (2) capacity for self-renewal, and (3) potential to differentiate into all hematopoietic lineages.[55] In adoptive transfer BM rescue experiments designed to remove the more primitive BM progenitors, DT_{390}-mIL-3 treatment inhibited the ability of BM to rescue lethally irradiated recipients that died within the first 2 to 3 weeks, but the effect could be overcome by increasing the dose of treated BM cells. Others believe that

Table 4. BM From Competitively Repopulated Mice Can Be Adoptively Transferred to Secondary Recipients

	% Positive Cells	
	Ly5.1	Ly5.2
Nontreated	60 ± 1.7	40 ± 1.7
DT_{390}-mIL-3	67 ± 4.9*	33 ± 4.9*

BM cells (8×10^6) from the DT_{390}-mIL-3–treated mice in experiment 1 were transplanted into C57BL/6 lethally irradiated (9.0 Gy) secondary recipients (n = 10/group) on day 110 post-BMT. Recipients were studied by flow cytometry on day 21 post-BMT for the presence of Ly5.1- or Ly5.2-expressing cells in the blood as described. Data are expressed as a mean percentage of positive cells ± 1 standard error of the mean.

* $P < .001$ compared with untreated controls.

early engraftment of donor cells that provide a radioprotective ability is a function of primitive progenitor cells that are more differentiated than PHSC. Radioprotective cells were found in the same fraction as the CFU-S, suggesting that they could represent cells at comparable stages of development.[50] Ogata et al[10] reported that the radioprotective ability of IL-3RAA$^-$ cells was significantly higher than that of the IL-3RAA$^+$ cell. Taken together with our results, these findings indicate that more primitive progenitor cells are heterogeneous in their expression of IL-3R numbers.

To test the effect of DT_{390}-mIL-3 on the PHSC, we further examined the long-term repopulating and self-renewal abilities of the treated BM cells using competitive repopulation assays. We used a 10 nmol/L DT_{390}-mIL-3 treatment for 8 hours because higher concentrations killed nonspecifically and longer incubations risked the survival of even nontreated cultured BM. Our competitive repopulation experiments showed that the percentage of long-term repopulating peripheral blood cells are proportional to the ratio of mixed congenic cells administered for BMT even though 10 million donor cells were injected. Even with the injection of such a high number of cells, the proportions of reconstituting progenitor cells correlated to the original proportion of donor BM cells, indicating that the relative effects of DT_{390}-mIL-3 on stem cells could be determined.

The studies in this report showed that congenic Ly5.2-marked stem cells were significantly inhibited, but not totally eliminated, by DT_{390}-mIL-3 treatment in either the primary recipients 95 days post-BMT or the secondary recipients 21 days postsecondary transfer. It is possible that the failure to eliminate all treated stem cells and entirely prevent long-term alloengraftment of Ly5.2 cells could be related to a failure of DT_{390}-mIL-3 to adequately kill these stem cells in the preincubation period. However, the treated Ly5.2 BM used in these experiments was simultaneously evaluated in colony assays and found to inhibited by 95% for CFU-GM, indicating that DT_{390}-mIL-3 was indeed highly cytotoxic. A more likely explanation is that some but not all PHSC express IL-3R, perhaps related to differences in expression relative to the stages of differentiation. Perhaps IL-3R is upregulated on more highly differentiated cells, as illustrated by our in vitro colony data. Once committed to myeloid lineages, the IL-3R may be continuously present on these progenitor cells until the end stages of differentiation.

Will DT_{390}-mIL-3 be useful for leukemia treatment? Murine and human myeloid and lymphoid leukemic cells display receptors for growth factors including IL-3, and many have been shown to be responsive to exogenous IL-3 in vitro.[3,4] The potency and specificity of DT_{390}-mIL-3 is high enough, with an IC_{50} of 0.025 nmol/L as compared with other fusion toxins[29-33] to render it an effective killing agent if it is not limited by its effects against progenitor cells. Our studies are encouraging in that, even when Ly5.2 stem cells were treated with DT_{390}-mIL-3, transplanted PHSC engrafted long-term and still had capacity for self-renewal when adoptively transferred into secondary recipients. One interpretation of these findings is that not all of the PHSC express IL-3R and that those that do not express the receptor escape killing. If this is the case, then DT_{390}-mIL-3 can be used for leukemia treatment or as an agent for purging leukemia cells before autologous BM transplant as long as sufficient quantities of treated BM cells are administered for an effective BM rescue.

In conclusion, the primary purpose of these studies was to determine the reactivity of a novel fusion protein, DT_{390}-mIL-3, with BM progenitors. Our data show that the agent was more effective in killing committed BM progenitor cells than primitive progenitor cells, possibly related to higher levels of IL-3R expression on these cells. The fact that DT_{390}-mIL-3 was highly toxic to leukemia cells indicates that it has potential for purging cancer cells from myeloid BM leukemia grafts or even for in vivo conditioning to reduce minimal residual disease. However, future experiments must determine the differential sensitivity of BM progenitors as compared with leukemia progenitors. Future studies are warranted because alternative therapies are needed for acute nonlymphocytic leukemia, which still presents a serious clinical problem.

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Recombinant Toxins Containing Human Granulocyte-Macrophage Colony-Stimulating Factor and Either Pseudomonas Exotoxin or Diphtheria Toxin Kill Gastrointestinal Cancer and Leukemia Cells

By Robert J. Kreitman and Ira Pastan

The granulocyte-macrophage colony-stimulating factor receptor (GM-CSFR) is a potential target for toxin-directed therapy, because it is overexpressed on many leukemias and solid tumors and apparently not on stem cells. To investigate the potential therapeutic use of GM-CSF toxins, we fused human GM-CSF to truncated forms of either Pseudomonas exotoxin (PE) or diphtheria toxin (DT) and tested the cytotoxicity of the resulting GM-CSF–PE38KDEL and DT388–GM-CSF on human gastrointestinal (GI) carcinomas and leukemias. Toward gastric and colon cancer cell lines, GM-CSF–PE38KDEL was much more cytotoxic than DT388–GM-CSF, with IC_{50}s (concentration resulting in 50% inhibition of protein synthesis) of 0.5 to 10 ng/mL compared with 4 to 400 ng/mL, respectively. In contrast, toward leukemia lines and fresh bone marrow cells DT388–GM-CSF was more cytotoxic than GM-CSF–PE38KDEL. The cytotoxicity of both GM-CSF–PE38KDEL and DT388–GM-CSF toward the human cells was specific, because it could be competed by an excess of GM-CSF. Binding studies indicated that human GM-CSF receptors were present on all of the human GI and leukemic cell lines tested, at levels of 540 to 3,700 sites per cell (kd = 0.2 to 2 nmol/L), and the number of sites per cell did not correlate with the cell type. A similar pattern of cytotoxicity was found with recombinant immunotoxins binding to the transferrin receptor, in that anti-TFR(Fv)–PE38KDEL was much more cytotoxic than DT388–anti-TFR(Fv) toward GI cells, but both were similar in their cytotoxic activity toward leukemia cells. The fact that PE is more effective than DT in killing GI but not leukemic tumor cells targeted by GM-CSF indicates a fundamental difference in the way PE or DT gains access to the cytosol in these cells. GM-CSF–PE38KDEL and DT388–GM-CSF deserve further evaluation as possible treatments for selected tumors.

GRANULOCYTE-MACROPHAGE colony-stimulating factor (GM-CSF) is a cytokine responsible for the growth, differentiation, and functional enhancement of granulocytes and macrophages.[1-3] Human GM-CSF is 127 amino acids long and binds to hematopoietic cells via high-affinity (kd = 10 to 50 pmol/L) receptors composed of α and β subunits.[4-8] GM-CSF is used to attenuate the myelosuppressive effects of chemotherapy in the treatment of not only hematologic malignancies but also solid tumors,[9] and it has been shown that GM-CSF usually does not stimulate the growth of solid tumors.[10] However, like leukemias,[11] solid tumors, including renal, lung, breast, and gastrointestinal carcinomas, also express GM-CSF receptors (GM-CSFRs), at least the low affinity α component (kd = 0.7 to 2 nmol/L).[12-16]

Recent studies have shown that GM-CSFR is absent on the most immature hematopoietic progenitors but increases in expression during maturation.[17] This finding suggests that the GM-CSFR may be a useful target for recombinant toxins or immunotoxins, which contain a cell binding protein linked to a protein toxin. Murine GM-CSF has recently been fused to truncated diphtheria toxin and the resulting DT_{390}mGM-CSF was cytotoxic to murine GM-CSFR–bearing cells.[18] A chemical conjugate of human GM-CSF with saporin was shown to kill mouse cells transfected with the human receptor.[19] To determine the utility of GM-CSFR as a means to target human hematologic and solid tumors, we fused human GM-CSF to truncated forms of Pseudomonas exotoxin (PE) or diphtheria toxin (DT).

PE is a 66-kD protein that, like DT, kills cells by binding to a receptor, internalizing via a coated pit, translocating its active fragment into the cytosol, and enzymatically ADP-ribosylating elongation factor-2.[20,21] The x-ray crystallographic structure of PE indicates three major domains, and mutational analysis has elucidated which domains are responsible for the several steps necessary to kill cells.[22,23] Domain Ia, which is composed of amino acids 1 through 252, functions to bind the toxin to the PE receptor. Domain III (amino acids 400 through 613) contains the enzymatic activity that ADP-ribosylates EF2. Domain II (amino acids 253 through 364) undergoes proteolytic processing and is responsible for translocating to the cytosol the 37-kD carboxyl terminus of PE that contains the ADP ribosylating activity. DT also undergoes proteolytic processing,[24] but its amino terminus contains the ADP-ribosylating activity and is translocated to the cytosol. Accordingly, in chimeric DT-containing toxins, the ligand replaces the toxin's binding domain at the carboxyl terminus. Conversely, in PE-containing chimeric toxins, the ligand replaces the toxin's binding domain at the amino terminus. The truncated form of PE used in the present study ends in KDEL, which has been shown to improve the cytotoxicity of PE-containing toxins and to increase binding of the toxin fragment to the KDEL receptor, which probably transports it to the endoplasmic reticulum, where it can translocate to the cytosol.[25-27]

MATERIALS AND METHODS

Plasmid construction. The polymerase chain reaction (PCR) was performed using a PCR kit from Perkin Elmer Cetus (Norwalk, CT). Denaturation temperature was 94°C for 1 minute, annealing was at 55°C for 2 minutes, and polymerization was at 72°C for 3 minutes,

From the Laboratory of Molecular Biology, Division of Cancer Biology, National Cancer Institute, National Institutes of Health, Bethesda, MD.

Submitted July 1, 1996; accepted February 13, 1997.

Address reprint requests to Ira Pastan, MD, Laboratory of Molecular Biology, Division of Cancer Biology, National Cancer Institute, National Institutes of Health, 37/4E16, 37 Convent Dr MSC 4255, Bethesda, MD 20892.

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with 10 seconds of extension per cycle. Plasmids were sequenced using an Applied Biosystems Taq Dyedeoxy cycle-sequencing kit and an automated sequencer (Applied Biosystems, Foster City, CA). The cDNA encoding human GM-CSF and containing *Nde* I and *Hin*dIII restriction sites at the ends was obtained from a human spleen cDNA library (Clontech, Palo Alto, CA) using primers BK-134 (5′-gcc-tgc-agc-cat-atg-gca-ccc-gcc-cgc-tcg-ccc-agc-ccc-3′) and BK144 (3′-ctg-acg-acc-ctc-ggt-cag-gtc-ctc-att-cga-act-taa-gcc-5′). The 0.41-kb *Nde* I-*Hin*dIII fragment was then ligated into the 3.0-kb *Nde* I-*Hin*dIII fragment of pRKL4.[28] The resulting plasmid, pRKGM2, contained the reported GM-CSF–encoding sequence,[6] except for mutations of codons 83 (cac→cgc), 86 (cag→cgg), and 97 (gca→gcg). The mutations at codons 83 and 86 were repaired using revertant primers that separately amplified codons 1 through 87 and 79 through 127. Primers for the first amplification were BK-145 (5′-gga-gat-ata-cat-atg-gca-cca-gca-cga-tcg-cca-agc-cca-agc-acg-cag-ccc-t gg-3′) and BK-147 (3′-ggg-aac-tgg-tac-tac-cga-tcg-gtg-atg-ttc-gtc-gtg-5′), and for the second amplification were BK-146 (5′-atg-gct-agc-cac-tac-aag-cag-cac-tgc-cct-cca-acc-3′) and BK-148 (3′-ctg-acg-acc-ctc-ggt-cat-gtc-ctt-cga-aga-act-taa-5′). The two overlapping fragments were used as a template for amplification with BK-145 and BK-148. The 0.39-kb *Nde* I-*Hin*dIII fragment of the final amplification product was then ligated to the 4.5-kb fragment of pRKB3F,[29] resulting in pRKGM9K, which had the correct sequence encoding GM-CSF–PE38KDEL. The 0.39-kb *Nde* I-*Hin*dIII fragment from pRKGM9K was then ligated to the 4.2-kb *Nde* I-*Hin*dIII fragment of pVCDT1-IL2,[30] resulting in pRKDTGM, which encodes DT388–GM-CSF. To make HB9K, encoding anti-TFR(Fv)-PE38KDEL, the 0.35-kb *Nde* I-*Bam*HI and 0.35-kb *Bam*HI-*Hin*dIII fragments of plasmid pJBDT1-anti-TFR(Fv)[31] were ligated to the 4.1-kb *Nde* I-*Hin*dIII fragment of pRK749K.[32]

Protein expression and purification. The method for expressing pRKGM9K, pRKDTGM, pRKHB9K, and pJBDT1-anti-TFR(Fv) and purifying the respective recombinant toxins GM-CSF–PE38KDEL, DT388–GM-CSF, anti-TFR(Fv)-PE38KDEL, and DT388-anti-TFR(Fv) differed slightly from the protocol previously reported.[33] *Escherichia coli* BL21/λDE3 cells[34] were transformed with each plasmid and grown overnight on LB-ampicillin plates. The transformed cells were cultured in superbroth containing 5 g/L glucose, 1.4 mmol/L $MgSO_4$, and 100 μg/mL ampicillin. At an OD_{650} of 2 to 3.5, protein synthesis was induced for 90 to 120 minutes with 1 mmol/L isopropyl-B-D-thiogalactopyranoside. The harvested cell paste was resuspended using a Tissuemizer tip (Thomas, Swedesboro, NJ) in TES buffer (50 mmol/L Tris, pH 8, 100 mmol/L NaCl, and 20 mmol/L EDTA) containing 180 μg/mL lysozyme. After incubating at 22°C for 1 hour, the cells were resuspended again and centrifuged at 27,000*g* for 50 minutes. The pellet was washed by resuspension and centrifugation three or four times with TES buffer containing 2.5% Triton-X-100 and then four times with TES. The inclusion bodies were resuspended in 5 to 10 mL of denaturation buffer (7 mol/L guanidine:HCl, 0.1 mol/L Tris, pH 8.0, and 5 mmol/L EDTA) by sonication or tissuemizing and diluted to a protein concentration of 10 mg/mL. The protein was reduced with dithioerythritol (65 mmol/L) for 4 to 24 hours at 22°C and rapidly diluted in a thin stream into refolding buffer (0.1 mol/L Tris, pH 8.0, 0.5 mol/L arginine:HCl, 2 mmol/L EDTA, and 0.9 mmol/L oxidized glutathione). After incubating at 10°C for 36 to 72 hours, the refolding buffer was either diluted 10-fold with water or dialyzed against 0.02 mol/L Tris, pH 7.4, 1 mmol/L EDTA, and 0.1 mol/L urea. The filtered protein was then purified by Qsepharose and MonoQ (Pharmacia, Piscataway, NJ) anion exchange and finally by sizing chromatography. The yield of purified active monomeric protein was 7.5% to 10% of total denatured recombinant protein.

Cytotoxicity assay. N87 gastric carcinoma cells were obtained from Dr R. King (Georgetown University, Washington, DC),[35] HUT-102 adult T-cell leukemia (ATL) cells were obtained from Dr T. Waldmann (National Institutes of Health), and the other cell lines were available from ATCC (Rockville, MD). A total of 1.5×10^4 cells/well were plated in 96-well plates; 24 hours later, toxin or control molecules were added and incubated for 48 hours in final volumes of 200 μL. The cells were pulsed for 4 to 6 hours with [^{3}H]-leucine 1 μCi/well, harvested, and counted. Bone marrow mononuclear cells were obtained from a patient with lymphocytic leukemia and from a normal donor for allogeneic bone marrow transplantation by Ficoll centrifugation, as described.[36] The marrow mononuclear cells (0.4 to 1 × 10^6/well) were incubated for 60 hours with toxin or control molecules in 100-μL aliquots of media consisting of 88% leucine-free RPMI, 2% RPMI, and 10% fetal bovine serum. The cells were then pulsed for 6 to 8 hours with [^{3}H]-leucine at 2 μCi/well, harvested, and counted. The IC_{50} was the concentration of toxin required for 50% protein synthesis inhibition.

Binding assay. Clinical grade GM-CSF was purchased from Immunex (Seattle, WA) and desalted on a PD-10 column (Pharmacia, Piscataway, NJ), equilibrated, and eluted with phosphate-buffered saline (PBS). Na ^{125}I (1 mCi; Amersham, Arlington Heights, IL) was added to a 100 μL volume of PBS containing GM-CSF (50 μg), sodium phosphate, pH 7.5 (150 mmol/L), and chloramine T (3.3 μg). After incubating for 2 minutes at 22°C, sodium metabisulfite (83 μg) was added and the [^{125}I]–GM-CSF purified on a PD-10 (Pharmacia) column equilibrated and eluted with 0.2% human serum albumin in PBS. HB21 (also termed anti-TFR–IgG), the monoclonal antibody to the transferrin receptor,[37] was labeled the same way except using 75 μg of anti-TFR–IgG and 10 μg of chloramine T. To determine the number of GM-CSFR sites per cell, cells were incubated in 96-well U-bottom plates in binding buffer (RPMI containing 0.1% bovine serum albumin and 0.2% NaN_3) containing increasing concentrations of [^{125}I]–GM-CSF with or without a 100-fold excess of unlabeled GM-CSF. After 30 minutes at 37°C, the cells were centrifuged and washed once with binding buffer and then counted. This method was easier and more reproducible than centrifuging the cells through n-butyl phthalate and counting the cell pellets. To determine the binding affinity of unlabeled toxins relative to that of GM-CSF, U937 cells (4.8 × 10^6/well) were plated in U-bottom 96-well plates in binding buffer and incubated with 1.2 nmol/L [^{125}I]–GM-CSF with and without different concentrations of re-

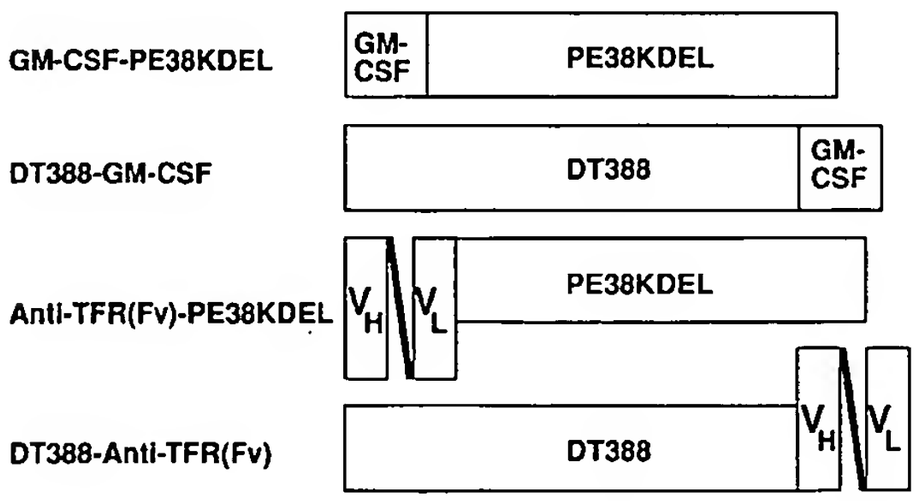

Fig 1. Schematic diagram of the recombinant toxins used. GM-CSF–PE38KDEL, encoded by pRKGM9K, contains the 127 amino acids of human GM-CSF followed by amino acids 253 through 364 and 381 through 608 of PE and then the sequence KDEL. DT388–GM-CSF, encoded by pRKDTGM, contains the first 388 amino acids of DT followed by human GM-CSF. Anti-TFR(Fv)–PE38KDEL and DT388–anti-TFR(Fv), encoded by pRKHB9K and pJBDT1–anti-TFR(Fv), respectively, contain the same toxin domains as the respective GM-CSF toxins, but the ligand is the single-chain Fv of an antitransferrin receptor antibody.

Table 1. Cytotoxicity of Recombinant Toxins Containing Human GM-CSF

Cell Line	Cell Type	IC_{50} (ng/mL) ± SD	
		GM-CSF–PE38KDEL	DT388–GM-CSF
LS174T	Colon	2.2 ± 0.7	70 ± 18
SW403	Colon	0.9 ± 0.5	15 ± 0.6
N87	Gastric	0.45 ± 0.2	3.7 ± 0.1
HTB-103	Gastric	9.5 ± 7.5	400 ± 300
HL60	Promyelocytic	> 100	0.4 ± 0.2
TF-1	Erythroleukemia	22 ± 8	0.02 ± 0.01
U937	Monocytic	9.5 ± 5.5	0.04 ± 0.02

Cells were plated in 96-well plates at 1.5×10^4/well and incubated at 37°C for 24 hours. The cells were then incubated with recombinant toxins for 24 to 48 hours and [^{3}H]-leucine for 4 to 6 hours.

combinant toxins. The relative binding affinity of the immunotoxins containing anti-TFR(Fv) was determined similarly using HUT-102 cells (8×10^5/well) and 0.1 nmol/L [^{125}I]—anti-TFR–IgG.

RESULTS

The fact that GM-CSFR is overexpressed on solid tumors and leukemia cells but is undetectable on the earliest hematopoietic progenitor cells[11-17] makes the GM-CSFR a potential target molecule. To determine whether GM-CSF can direct bacterial toxins to kill GM-CSF–expressing tumor cells, we fused GM-CSF to truncated forms of PE or DT and tested the resulting fusion toxins for cytotoxicity and binding.

Preparation of recombinant GM-CSF toxins. Figure 1 shows schematic diagrams of GM-CSF–PE38KDEL and DT388–GM-CSF. As confirmed by the DNA sequence analysis, the first molecule contains the 127 amino acid human GM-CSF ligand at the amino terminus of the truncated toxin, which consists of amino acids 253 through 364 and 381 through 608 of PE, followed by the KDEL carboxyl terminus. DT388–GM-CSF contains the first 388 amino acids of DT followed by human GM-CSF. GM-CSF was placed at the amino terminus of truncated PE and at the carboxyl terminus of truncated DT to replace the binding domains that are normally present in those positions and because a free carboxyl terminus of PE and a free amino terminus of DT is necessary for cytotoxicity.[30,38] To properly fold the recombinant toxins, each of which contains 6 cysteine residues, the insoluble inclusion body protein was denatured, reduced, and refolded in a redox buffer as described in Materials and Methods. Each protein could be purified to near homogeneity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (gel not shown) in a yield of 10% of the total recombinant protein renatured. The purified recombinant toxins were then tested for cytotoxicity and binding.

Cytotoxicity of GM-CSF toxins. To determine the sensitivity of leukemia and GI carcinomas to GM-CSF toxins, the cells were incubated with recombinant toxins and [^{3}H]-leucine incorporation was measured. The IC_{50}s, the concentrations of toxin necessary for 50% inhibition of protein synthesis, are listed in Table 1. For GM-CSF–PE38KDEL, the IC_{50}s on GI carcinomas ranged from 0.45 ng/mL on N87 to 9.5 ng/mL on HTB-103 cells. Leukemia cells were less sensitive to GM-CSF–PE38KDEL, with IC_{50}s ranging from 9.5 ng/mL on U937 monocytic leukemia cells to greater than 100 ng/mL on HL60 promyelocytic leukemia cells. In contrast, DT388–GM-CSF was much more cytotoxic to leukemia cells than to GI carcinomas, with IC_{50}s ranging from 0.02 ng/mL to 0.4 ng/mL on the leukemia lines compared with 3.7 ng/mL to 400 ng/mL on the GI carcinoma lines. Thus, GM-CSF–PE38KDEL was much more cytotoxic than DT388–GM-CSF on the GI carcinoma lines, whereas the reverse was true on the leukemia lines.

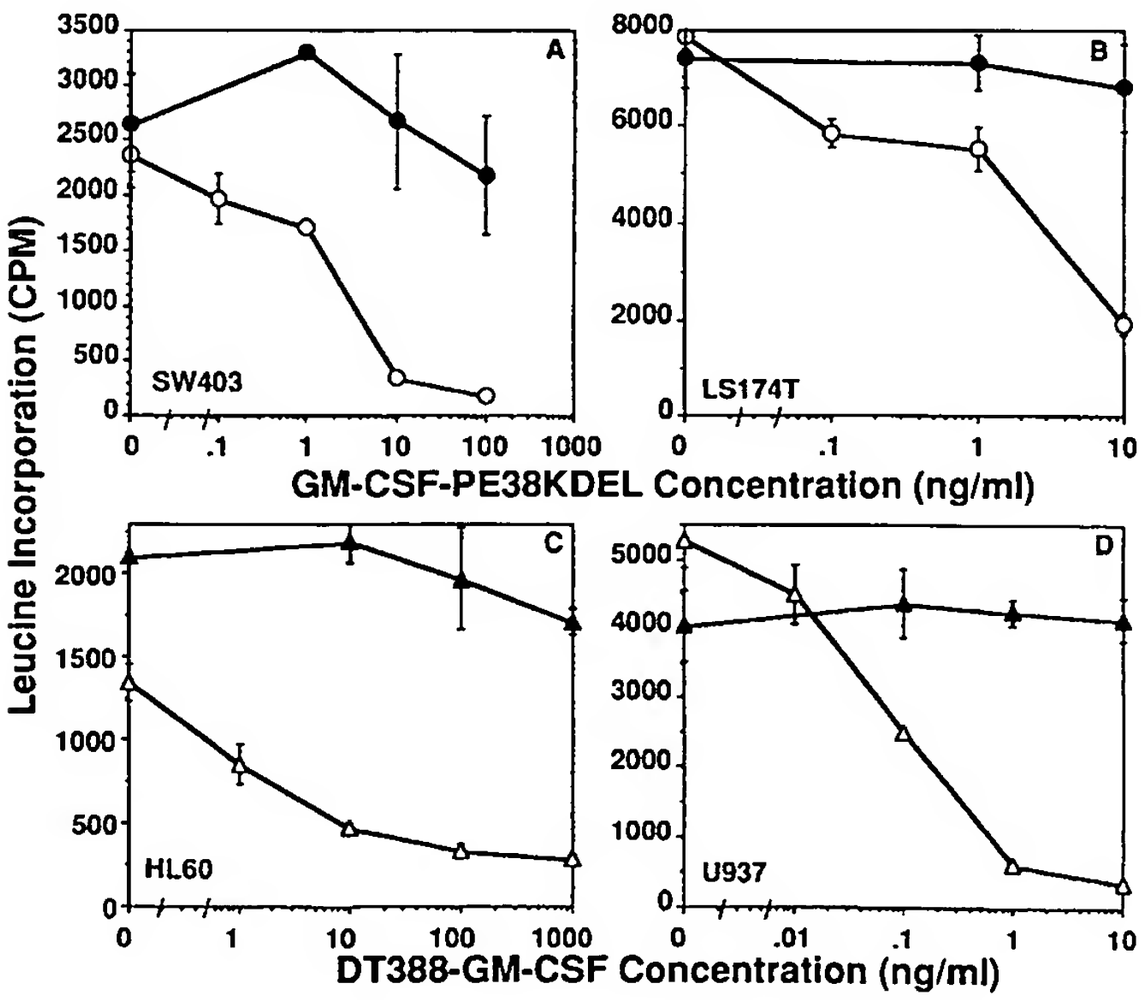

Fig 2. Cytotoxicity and specificity of GM-CSF toxins on target cells. The colon lines SW403 (A) and LS174T (B) were incubated with GM-CSF–PE38KDEL and DT388–GM-CSF was incubated with the leukemia lines HL60 (C) and U937 (D) in the presence (●, ▲) or absence (○, △) of 5 μg/mL of GM-CSF. The cells were pulsed with [^{3}H]-leucine and harvested, and the leucine incorporation was determined.

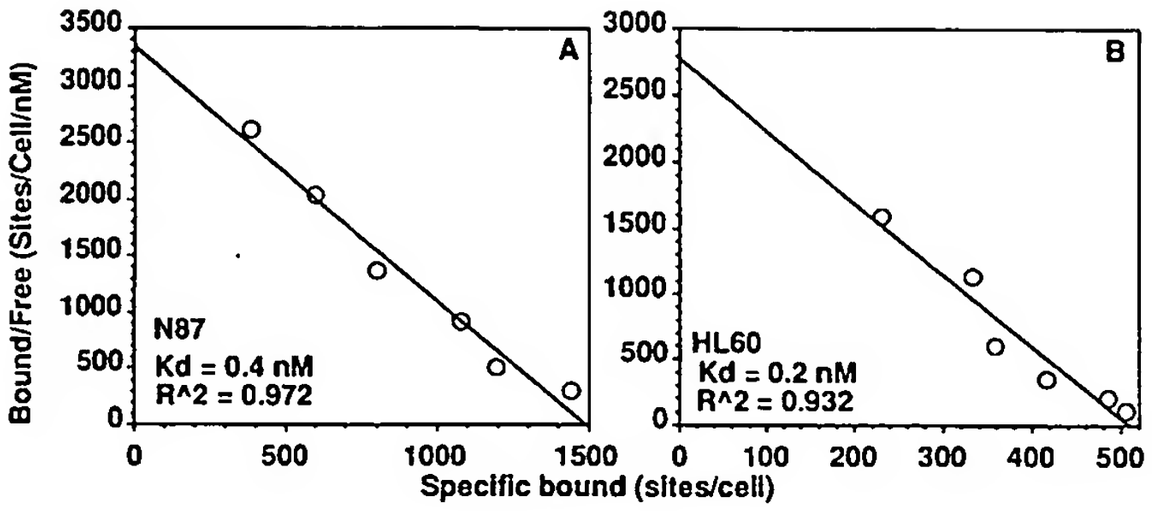

Fig 3. Scatchard plots of [^{125}I]–GM-CSF binding to target cells. In (A), N87 cells (1.3 × 10^6/200 μL/well) were incubated with 0.15, 0.3, 0.6, 1.2, 2.4, and 4.8 nmol/L [^{125}I]–GM-CSF (5.7 μCi/μg) in RPMI containing 0.1% bovine serum albumin and 0.2% NaN_3 for 30 minutes, washed, and counted. HL60 cells (B) were assayed similarly at 2.2 × 10^6/well.

Cytotoxic specificity of the recombinant toxins containing GM-CSF. To determine whether the cytotoxicity of the recombinant GM-CSF toxins was specific in requiring internalization through the GM-CSF receptor, cytotoxicity experiments were performed where the binding of the chimeric toxins was competed by an excess of GM-CSF (2.5 to 5 μg/mL). Figure 2 shows representative cytotoxicity curves for GM-CSF–PE38KDEL and DT388–GM-CSF in the presence and absence of an excess of GM-CSF. It was found that an excess of GM-CSF prevented the cytotoxic activity of both recombinant toxins, indicating that their cytotoxic activity required binding to the GM-CSF receptor. Such specificity was also shown by GM-CSF–PE38KDEL for N87, HTB-103, TF-1, and U937 cells and by DT388–GM-CSF for TF-1, LS174T, and SW403 cells (data not shown). To determine whether the cytotoxicity of the GM-CSF toxins was only due to their binding to the GM-CSFR and did not require the action of the toxin domains, the cells were incubated with and without 5 μg/mL of GM-CSF. In Fig 2, the points on the y-axis of each curve, where the toxin concentration equals 0, show the leucine incorporation in cells with and without 5 μg/mL of GM-CSF. In none of the seven cell lines did GM-CSF alone result in greater than 50% inhibition of protein synthesis. Thus, the cytotoxicity of GM-CSF–PE38KDEL and DT388–GM-CSF required both binding to the GM-CSFR and also internalization and action of the toxin domains. Interestingly, the leukemic cell lines HL60 (Fig 2C) and TF-1 (data not shown) showed significant (but <twofold) stimulation with 5 μg/mL of GM-CSF in the absence of toxin, but the solid tumors showed no significant stimulatory response to GM-CSF.

Quantitation of GM-CSFR on the target cells. The number of GM-CSFR sites per cell were quantitated by radiolabeled binding assay using [^{125}I]–GM-CSF on the cell lines to determine whether cytotoxic activity correlated with receptor expression. Representative Scatchard plots are shown in Fig 3. The binding assay was designed to determine the number of low-affinity sites, which outnumber the small number of high-affinity sites.[4-8] As shown in Table 2, the number of GM-CSFR sites per cell varied somewhat from assay to assay, but the average values varied from 500 sites per cell for HTB-103 gastric cells to 3,700 sites per cell for TF-1 cells. The kds for the seven cell lines ranged from 0.2 to 1.9 nmol/L, consistent with low-affinity binding sites. Together with the cytotoxicity data from Table 1, it can be seen that cytotoxicity did correlate with the number of GM-CSFR sites per cell when examining one type of cell at a time. For example, for either toxin toward the GI carcinoma lines, SW403 and N87 were more sensitive than LS174T and HTB-103 was least sensitive, matching the order of their GM-CSFR expression. Also, of the three leukemia lines, HL60 had the least numbers of sites per cell and was less sensitive to either toxin than were TF-1 or U937 cells. However, GM-CSF–PE38KDEL was more cytotoxic toward the GI lines than the leukemia lines, and DT388–GM-CSF was more cytotoxic toward the leukemia lines than the GI lines. Thus, for both recombinant toxins the difference in their cytotoxic activity toward leukemia and solid tumor cells was likely due to fundamental differences between these cell types unrelated to the numbers of receptors expressed.

Sensitivity of fresh bone marrow cells to GM-CSF toxins. To determine if the data on cell lines from patients with leukemia and GI cancer would apply to fresh human hematopoietic cells, bone marrow mononuclear cells from two donors were partially purified by Ficoll centrifugation and incubated with the recombinant toxins. The first sample was taken from normal marrow and the second from a patient whose marrow contained normal hematopoietic progenitor cells and was 50% involved with a B-cell small-cell lymphoma. As shown in Table 3, the marrow mononuclear cells were much more sensitive to DT388–GM-CSF compared with GM-CSF–PE38KDEL. In the normal marrow sample, the IC_{50} for DT388–GM-CSF was 2.1 ng/mL, compared with greater than 1,000 ng/mL for GM-CSF–

Table 2. Expression of GM-CSFR on Human Cells

Cell Line	Sites/Cell	kd (nmol/L)
LS174T	900 ± 700	1.7 ± 1
SW403	3,050 ± 900	1.6 ± 0.5
N87	2,400 ± 1,200	0.9 ± 0.7
HTB-103	500 ± 200	0.4 ± 0.4
HL60	540 ± 50	0.2 ± 0.01
TF-1	3,700 ± 1,500	0.5 ± 0.05
U937	3,500 ± 350	1.9 ± 0.2

Cells were incubated at 37°C for 30 minutes with [^{125}I]–GM-CSF with or without a 100-fold excess of GM-CSF in RPMI media containing 1 mg/mL bovine serum albumin and 0.2% NaN_3. Unbound GM-CSF was removed from the cells either by centrifuging through n-butyl phthalate or by centrifuging and washing the cells with binding buffer.

Table 3. Sensitivity of Fresh Hematopoietic Cells to Recombinant GM-CSF Toxins

Sample	IC50 (ng/mL)	
	GM-CSF–PE38KDEL	DT388–GM-CSF
Normal bone marrow	>1,000	2.1
Marrow in lymphoma patient	>100	0.9
Normal peripheral lymphocytes	>1,000	>1,000
Peripheral blood B leukemia	>1,000	>1,000

Samples consisted of mononuclear cells obtained by Ficoll centrifugation and were incubated with recombinant toxins for 60 hours followed by [^{3}H]-leucine for 4 to 6 hours.

Table 4. Sensitivity of Colon and Leukemia Lines to Recombinant Immunotoxins Binding to the Human Transferrin Receptor

Cell Line	Cell Type	IC_{50} (ng/mL) ± SD	
		Anti-TFR(Fv)–PE38KDEL	DT388–Anti-TFR(Fv)
LS174T	Colon	0.008 ± 0.003	0.4 ± 0.05
SW403	Colon	0.02 ± 0.007	0.7 ± 0.3
N87	Gastric	0.003 ± 0.0004	0.2 ± 0.07
HTB-103	Gastric	0.002 ± 0.0006	0.2 ± 0.06
HL60	Promyelocytic	0.4 ± 0.15	0.2 ± 0.015
TF-1	Erythroleukemia	0.018 ± 0.006	0.13 ± 0.1
U937	Monocytic	0.1 ± 0.09	0.13 ± 0.08

PE38KDEL. The results were similar in the marrow contaminated with malignant B cells, with an IC_{50} of 0.9 ng/mL for DT388–GM-CSF and greater than 100 ng/mL for GM-CSF–PE38KDEL. Although these marrow samples contained differentiated cells, the cytotoxicity of DT388–GM-CSF appeared directed toward the hematopoietic progenitor cells, because fresh normal or malignant lymphocytes isolated from the peripheral blood were resistant (Table 3). To determine whether the cytotoxic activity of GM-CSF toxin towards the marrow cells was specific in requiring binding to the GM-CSFR, we simultaneously tested recombinant toxin containing PE38KDEL or DT388 but not GM-CSF. As shown in Fig 4A, PE38KDEL alone[39] or DT388-IL2[30] showed no cytotoxic activity toward the normal marrow cells, and PE38KDEL was significantly less cytotoxic than GM-CSF–PE38KDEL. Thus, the cytotoxic activity of the recombinant toxins containing GM-CSF was not due to nonspecific internalization into the fresh marrow cells. As shown by the Y-axis of Fig 4B, in the absence of toxin, 20 ng/mL of GM-CSF resulted in a 25% increase in protein synthesis, indicating that the cytotoxic activity of the GM-CSF toxin was not only due to their binding to the GM-CSFR on the cells but also required action of the bacterial toxins after internalization. Finally, Fig 4B shows that the cytotoxic activity of DT388–GM-CSF could be competed by 20 ng/mL of GM-CSF, confirming that its cytotoxic activity required binding to the GM-CSFR on the fresh human hematopoietic progenitor cells.

Sensitivity of GI and leukemia cells to toxins carrying another ligand. To determine whether the difference in sensitivity of GI and leukemia cells to recombinant toxins was due to differences in the way the cells handle the GM-CSFR or due to differences between these cells that are unrelated to the GM-CSFR, they were incubated with recombinant toxins containing a different ligand. The ligand chosen was anti-TFR(Fv), which binds to the human transferrin receptor.[31] As shown in Fig 1, anti-TFR(Fv)–PE38KDEL contains the exact same toxin domains as GM-CSF–PE38KDEL, and DT388–anti-TFR(Fv) contains the exact same toxin domains as DT388–GM-CSF. Table 4 lists the IC_{50}s of these two recombinant immunotoxins toward the GI and leukemia cell lines. It can be seen that anti-TFR(Fv)–PE38KDEL was usually much more cytotoxic to the GI cell lines than the leukemia cell lines, with differences being as great as 200-fold. In contrast, DT388–anti-TFR(Fv) was usually more cytotoxic to the leukemia cells than to the GI carcinoma cells, with the differences being less than 10-fold. Thus, regardless of the targeting ligand, GI carcinomas were more sensitive than leukemias to PE38KDEL, and leukemias were more sensitive than GI carcinomas to DT388.

Assessment of the relative affinities of the recombinant toxins. To compare the cytotoxic activities of GM-CSF–PE38KDEL or anti-TFR(Fv)–PE38KDEL with those of DT388–GM-CSF and DT388–anti-TFR(Fv), respectively, one must take into consideration quantitative differences in the binding of the ligands, depending on whether the ligand is located at the amino or carboxyl terminus of the toxin. To determine the relative binding affinity of the recombinant toxins, they were tested for their ability to displace radiolabeled ligand. In Fig 5A, the amount of [^{125}I]–GM-CSF bound to U937 cells is shown as a function of increasing

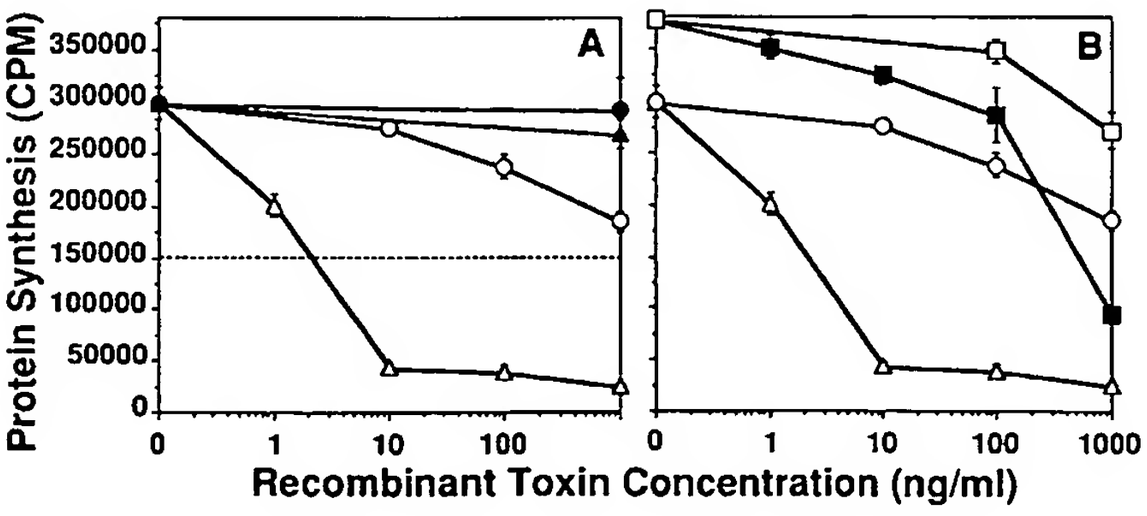

Fig 4. Cytotoxicity of recombinant toxins towards normal bone marrow cells. Normal marrow mononuclear cells (10^6/0.1 mL well) were incubated with DT388–GM-CSF (△) or GM-CSF–PE38KDEL (○) for 60 hours and pulsed with [^{3}H]-leucine. In (A), the cells were incubated in parallel with the negative control molecules PE38KDEL (●) or DT388-IL2 (▲). In (B), the cells were also incubated with DT388–GM-CSF (■) or GM-CSF–PE38KDEL (□) in the presence of 20 ng/mL of GM-CSF.

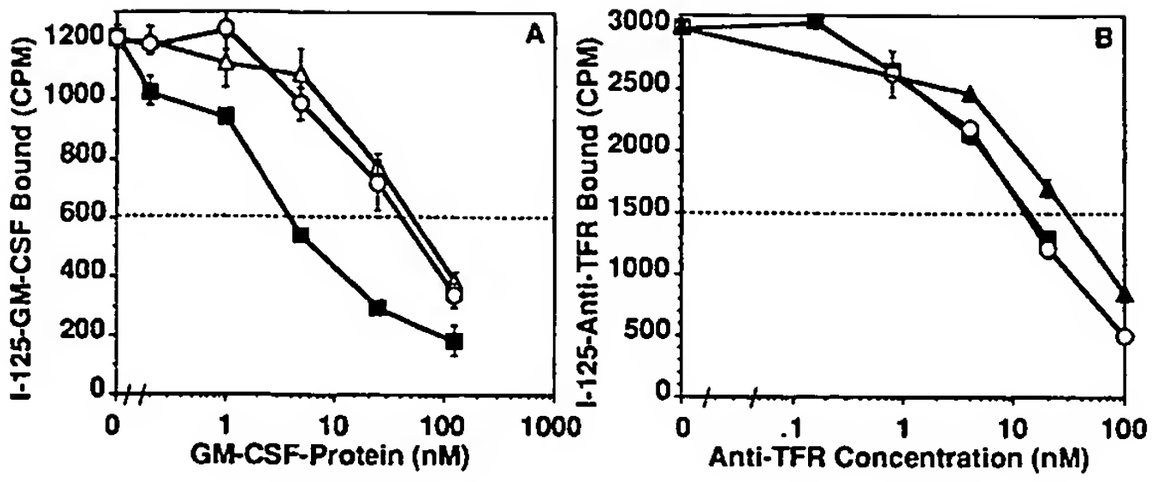

Fig 5. Displacement analysis of recombinant toxins. In (A), U937 cells (4.5×10^6/well) were incubated with [^{125}I]–GM-CSF and the indicated concentrations of GM-CSF (■), GM-CSF–PE38KDEL (○), or DT388–GM-CSF (△). In (B), HUT-102 cells (8×10^5/well) were incubated with [^{125}I]—anti-TFR–IgG and the indicated concentrations of anti-TFR–IgG (■), anti-TFR(Fv)–PE38KDEL (○), or DT388–anti-TFR(Fv) (▲). The cells were harvested and counted as in Fig 3.

concentrations of either GM-CSF, GM-CSF–PE38KDEL, or DT388–GM-CSF. The EC_{50}, the concentration necessary for 50% competition of [^{125}I]–GM-CSF binding, was 3.9 nmol/L for GM-CSF, 41 nmol/L for GM-CSF–PE38KDEL, and 50 nmol/L for DT388–GM-CSF. In Fig 5B, the amount of [^{125}I]—anti-TFR–IgG binding to HUT-102 cells is shown as a function of increasing concentrations of either anti-TFR–IgG, anti-TFR(Fv)–PE38KDEL, or DT388–anti-TFR(Fv). The EC_{50} was 13 nmol/L for anti-TFR–IgG, 12 nmol/L for anti-TFR(Fv)–PE38KDEL, and 29 nmol/L for DT388–anti-TFR(Fv). Table 5 was then constructed to display the IC_{50}s for the recombinant toxins corrected for binding affinity. Lower values of IC_{50}/EC_{50} would indicate more efficient killing of cells due to processes occurring after binding. Table 5 indicates that the IC_{50}/EC_{50} ratios of GM-CSF–PE38KDEL are much less than those of DT388–GM-CSF toward GI carcinomas, whereas the reverse was true for leukemias. Similarly, the IC_{50}/EC_{50} ratios of anti-TFR(Fv)–PE38KDEL are much less than those of DT388–anti-TFR(Fv) toward GI carcinomas, but not toward leukemias. Thus, regardless of the targeting ligand, PE38KDEL was more cytotoxic toward solid tumors and DT388 was more cytotoxic toward leukemias due to processes in those cells that occur after receptor binding.

DISCUSSION

To determine the sensitivity of solid tumors and leukemias to recombinant toxins containing GM-CSF, we tested the sensitivity of such cell lines to GM-CSF–PE38KDEL and DT388–GM-CSF. We found that, although both recombinant toxins were specifically cytotoxic to both types of cells, solid tumor cells were more sensitive to GM-CSF–PE38KDEL and leukemia cells were more sensitive to DT388–GM-CSF. This was not a phenomenon peculiar to cell lines, because fresh human marrow progenitor cells behaved similar to the leukemia lines. Based on a similar pattern of cytotoxicity using the same toxins targeted to the transferrin receptor, it appears that these differences are due to inherent differences between solid tumors and leukemias in the way by which they handle the toxins intracellularly.

Table 5. Cytotoxicity of Recombinant Toxins Corrected for Binding Affinity

	IC_{50} (pmol/L)/EC_{50} (nmol/L)			
Cell Line	GM-CSF–PE38KDEL	DT388–GM-CSF	Anti-TFR(Fv)–PE38KDEL	DT388–Anti-TFR(Fv)
LS174T	1	25	0.003	0.2
SW403	0.4	5	0.007	0.35
N87	0.2	1.3	0.001	0.1
HTB-103	4	140	0.0007	0.1
HL60	>45	0.14	0.14	0.1
TF-1	10	0.007	0.006	0.07
U937	4	0.014	0.035	0.07

The IC_{50}s shown in Tables 1 and 4 were converted to picomoles per liter and divided by the EC_{50} as shown in Fig 5.

It is believed that cytotoxicity by PE requires proteolytic processing by Furin between amino acids 279 and 280 and that the carboxy terminal fragment is transported by the KDEL receptor from at least the transreticular Golgi toward the endoplasmic reticulum, from which it translocates to the cytosol.[25,38,40,41] DT is also cleaved by Furin between arginine 193 and serine 194, and arginine 193 at the carboxyl terminus of the DT fragment A is necessary for translocation.[24,40] However, this fragment is thought to translocate from the endosome to the cytosol via membrane insertion and passage through ion-conductive channels.[42-44] Because both PE and DT are processed by the same enzyme, both toxins ultimately ADP ribosylate EF2 in the cytosol, and in both PE38KDEL and DT388 ADP ribosylation activity is not altered by the ligand to which the toxin is fused (data not shown). Thus, the difference in activity between GM-CSF–PE38KDEL and DT388–GM-CSF would not be due to differences in ADP-ribosylation activity. We therefore speculate from our data that leukemic cells differ from GI carcinoma cells in the intracellular transport or translocation of protein molecules. For example, in leukemic cells, a protein molecule may have a higher chance of entering the cytosol if it can translocate directly from the endosome than if it must be transported intracellularly. Conversely, in solid tumors, proteins may not easily translocate from endosomes and may require transport to an organelle such as the endoplasmic reticulum that has preexisting pores. It is also possible that leukemic and solid tumor cells differ in the function and location of lysosomes, which may degrade toxin molecules before they reach the cytosol. So far, our data with cell lines are consistent with that on fresh cells from patients with GM-CSFR$^+$ leukemia (Rozemuller et al, manuscript submitted). Because one molecule of DT[45] or PE (Willingham and Pastan, unpublished data) in the cytosol appears sufficient to kill a cell, it is very

important to identify and attempt to overcome these potential impediments to translocation.

We have shown that human GM-CSF can be used to target truncated forms of either PE or DT to inhibit protein synthesis in receptor-bearing solid or hematopoietic tumor cells. Further development of these agents for the treatment of human tumors should include monkey toxicology studies, because murine GM-CSFR does not bind human GM-CSF.[46] Such studies could determine whether cytotoxicity toward GM-CSFR$^+$ normal cells spares stem cells and, if so, whether hematopoietic toxicity is low enough to allow the delivery of high doses. If so, such agents could prove useful clinically in the purging of bone marrow or stem cell autografts before transplantation or in the direct systemic treatment of human leukemia and solid tumors.

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Induction of Apoptosis in Multidrug-resistant and Radiation-resistant Acute Myeloid Leukemia Cells by a Recombinant Fusion Toxin Directed against the Human Granulocyte Macrophage Colony-stimulating Factor Receptor[1]

John P. Perentesis,[2] Kevin G. Waddick, Anne E. Bendel, Yu Shao, Berta E. Warman, Mridula Chandan-Langlie, and Fatih M. Uckun

Biotherapy Institute [J. P. P., K. G. W., Y. S., F. M. U.] and Departments of Pediatrics [J. P. P., A. E. B., Y. S., B. E. W., F. M.U.], Therapeutic Radiology-Radiation Oncology [K. G. W., Y. S., F. M. U.], Biochemistry [J. P. P.], and Pharmacology [F. M. U.], University of Minnesota Medical School and Academic Health Center, Minneapolis, Minnesota 55455, and Hughes Institute, St. Paul, Minnesota 55113 [M. C-L.]

ABSTRACT

Multiagent chemotherapy regimens fail to cure more than one-half of the patients with acute myeloid leukemia (AML) because of the emergence of dominant multidrug-resistant subclones of leukemia cells. We have developed a recombinant diphtheria toxin-human granulocyte macrophage colony-stimulating factor chimeric fusion protein (DT_{ct}GMCSF) that specifically targets GMCSF receptor-positive AML cells. This novel biotherapeutic agent induced rapid apoptotic cell death of chemotherapy-resistant AML cell lines and primary leukemic cells from treatment-refractory AML patients. Our results suggest that DT_{ct}GMCSF may be useful in the treatment of AML patients whose leukemia has recurred and developed resistance to contemporary chemotherapy programs.

INTRODUCTION

AML[3] is the most common form of acute leukemia in adults and the second most frequent leukemia in children (1, 2). Multiagent chemotherapy regimens fail to cure more than one-half of patients with AML because of the emergence of dominant multidrug-resistant subclones of leukemia cells (3). Myeloablative chemotherapy and supralethal radiochemotherapy followed by allogeneic or autologous bone marrow transplantation have been used in an attempt to overcome drug resistance in AML. These regimens are associated with considerable morbidity and mortality, frequently fail to eradicate multidrug-resistant or radiation-resistant leukemia cells, and have effected only modest improvements in the overall survival of AML patients, underscoring the need for rational drug design-based therapies for AML (4–6).

Native DT is a 535-residue protein secreted by *Corynebacterium diphtheriae* and one of the most toxic substances found in nature (7). A single DT molecule entering a human cell can induce catalytic inactivation of protein synthesis, leading to rapid cell death (7). DT inhibits protein synthesis by catalyzing the ADP-ribosylation and inactivation of elongation factor 2, an essential protein synthesis cofactor, at a highly conserved post-translationally modified histidine residue known as diphthamide (8). Its profound toxicity is a result of the catalytic nature of its mechanism of action, as well as the ubiquitous expression of its receptor, a heparin-binding EGF-like precursor, on human cells (9). Biochemical (10) and genetic (11–13) analyses of DT have provided tremendous insights into its structure-function relationships. These investigations have also guided the design of novel recombinant therapeutic agents that were constructed by genetic deletion of the receptor binding domain of the toxin and its replacement with growth factors, serving to effectively redirect the toxin to growth factor receptors found on malignant cells (14–18). In addition, recent X-ray crystallographic analyses (19, 20) have expanded upon these investigations to identify three functionally distinct domains within the DT structure: (*a*) an amino-terminal catalytic domain ("C" domain) that contains the ADP-ribosyltransferase active site; (*b*) a transmembrane domain ("T" domain) found in the middle of the protein and facilitating C domain translocation across membranes; and (*c*) a carboxyl-terminal receptor binding domain ("R" domain) that mediates binding and leads to receptor-mediated endocytosis. These structural studies have also revealed that DT amino acid residues 380–386, located in a small loop separating the receptor binding domain from the catalytic and transmembrane domains, allow the entire M_r 15,000 receptor binding domain to flexibly rotate as a unit by 180°, with atomic movement of up to

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[2] To whom requests for reprints should be addressed, at University of Minnesota Medical School, Box 422, 420 Delaware Street SE, Minneapolis, MN 55455. Phone: (612) 626-2902; Fax: (612) 626-4842.
[3] The abbreviations used are: AML, acute myeloid leukemia; DT, diphtheria toxin; GMCSF, granulocyte macrophage colony-stimulating factor; DT_{ct}GMCSF, DT-GMCSF chimeric fusion protein; GMCSF-R, GMCSF receptor; TCA, trichloroacetic acid; Pgp, P-glycoprotein; MDR, multidrug resistance; MRP, MDR-associated protein; IMDM, Iscove's modified Dulbecco's medium.

65 Å. Separate studies have elucidated the crystal structure of GMCSF (21, 22), demonstrated that it is a member of the four-helix bundle family of cytokines (23), and defined critical domains that are essential for high-affinity binding to its receptor (GMCSF-R).

We have built upon the investigations of the molecular and structural biology of DT and GMCSF to create a novel fusion toxin, DT_{ct}GMCSF, that preserves the catalytic and transmembrane domains of DT but replaces the native toxin receptor binding domain with human GMCSF and includes a sterically neutral peptide linker separating the toxin and growth factor domains at the site of the flexible peptide loop at residues 380–386. Here, we demonstrate that DT_{ct}GMCSF is able to redirect the protein synthesis inhibitory action of DT to multidrug-resistant as well as radiation-resistant AML cells, resulting in apoptotic cell death. The mechanism of action of DT_{ct}GMCSF is different from those of other antileukemic drugs used in contemporary AML therapy programs and may allow us to overcome drug and radiation resistance in AML.

MATERIALS AND METHODS

DT_{ct}GMCSF Fusion Toxin. The engineering and production of DT_{ct}GMCSF fusion toxin in highly purified form were described in detail in a previous report from our laboratory (24). In brief, DT_{ct}GMCSF is a 521-amino acid residue chimeric protein containing a predicted amino-terminal methionine residue, followed by amino acid residues 1–385 of DT, a Ser-$(Gly)_4$-Ser-Met linker peptide, and mature human GMCSF. This fusion toxin preserves the portions of DT, including the lethal catalytic ADP-ribosyltransferase domain (C domain) and the contiguous proximal portion of the toxin that is associated with translocation across cellular membranes (T domain). The native receptor binding domain of DT was completely deleted in the construction of the DT_{ct}-GMCSF fusion toxin. The short Ser-$(Gly)_4$-Ser-Met linker peptide was inserted in the fusion toxin at the natural DT receptor "hinge site" to separate the DT and GMCSF moieties and insure that the NH_2-terminal helicies of GMCSF would be accessible for high-affinity receptor binding. DT_{ct}GMCSF was expressed with high-efficiency fermentation methods in *Escherichia coli* and purified through sequential anti-DT immunoaffinity and mono-Q high-pressure liquid chromatographic methods, followed by endotoxin removal (24, 25).

Cell Lines and Culture Conditions. GMCSF-R-bearing human leukemia cell lines included the human acute promyelocytic leukemia cell line HL-60 (26) and the GMCSF-R-negative control leukemia cell line K562 (27) obtained from the American Type Culture Collection (Rockville, MD). Multidrug-resistant subclones of HL-60 cells, including HL-60/VCR cells (28), which express a P-glycoprotein associated MDR phenotype, and HL-60/ADR cells (29, 30), which express a MRP-associated MDR phenotype, were the gift of Dr. M. Center (Kansas State University, Manhattan, KS). HL-60 cells were maintained in IMDM, 20% FBS, 50 units/ml penicillin, and 50 μg/ml streptomycin. K562 cells were maintained in RPMI, 10% FBS, 50 units/ml penicillin, and 50 μg/ml streptomycin. Primary leukemic cells were obtained from the previously cryopreserved AML bone marrow samples of therapy-refractory patients stored in the liquid nitrogen tanks of the Children's Cancer Group Cell Bank at the University of Minnesota.

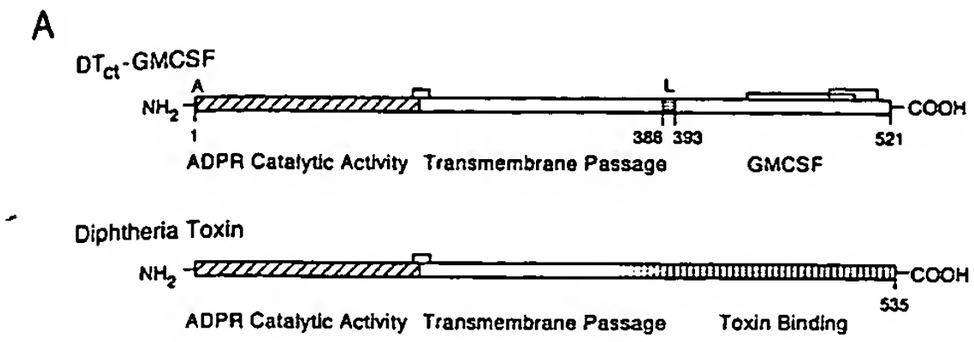
GMCSF Displacement Assay. Ligand displacement assays were performed as described previously (31). Radiolabeled ^{125}I-GMCSF (Dupont-NEN) at a concentration of 40 pM and unlabeled DT_{ct}GMCSF or recombinant human GMCSF at increasing concentrations (1 pM to 100 nM) were incubated at 4°C with 4×10^6 HL-60 myeloid leukemia cells in 400 μl of IMDM binding buffer. After a 2-h incubation, cells were transferred into 0.75 ml of an ice-cold mixture of 75% FBS in binding buffer, and the cells were then collected by centrifugation. A gamma counter was used to measure radioactivity in the supernatants and pellets.

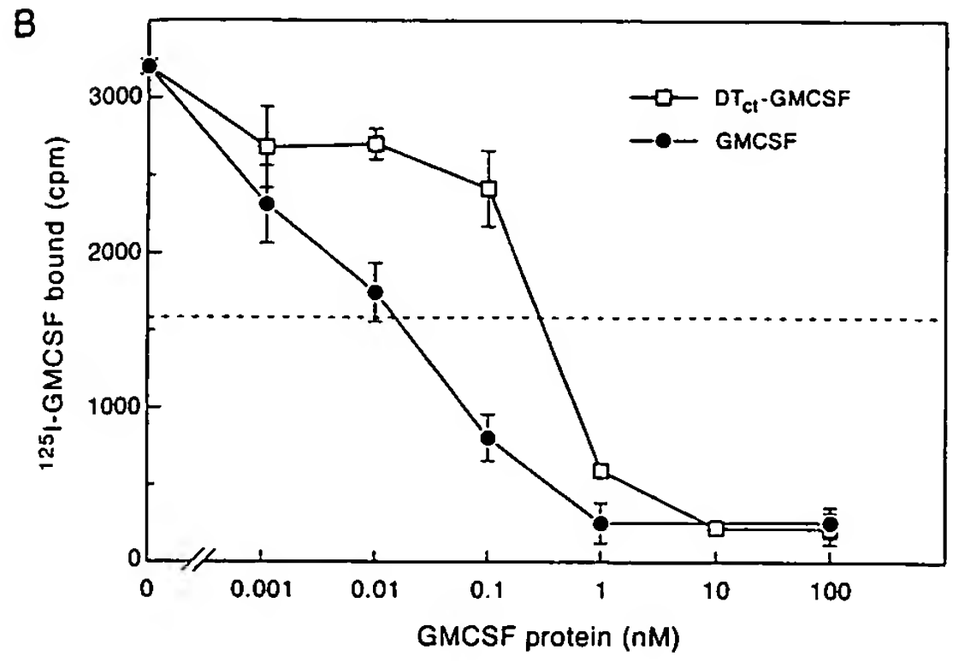
Protein Synthesis Inhibition Assays. For protein synthesis inhibition assays, 1×10^5 cells/well were seeded into 96-well sterile MultiScreen-HV plates (Millipore Corp.) containing leucine-free RPMI followed by the addition of DT_{ct}GMCSF, native recombinant human GMCSF (Immunex), or PBS, and incubated at 37°C for 24 h. RPMI plus [^{3}H]leucine (L-[3,4,5-^{3}H]; DuPont-NEN) was added to a final concentration of 1 μCi/well with a 2-h pulse incubation. Using a MultiScreen system vacuum manifold, the cells were washed twice with PBS and lysed with sterile water, and the insoluble protein was precipitated in 20% TCA. The insoluble protein was washed three times with 10% TCA, and radioisotope incorporation into protein as collected on the MultiScreen-HV plate filter was measured in a Beckman LS7000 scintillation counter after a 30-min incubation in 0.42% sodium hypochlorite. Chemiluminescent sodium hypochlorite controls were prepared daily.

Apoptosis Assays. To detect apoptotic changes, cells were harvested 24 h after continuous exposure to the DT_{ct}GMCSF fusion toxin, and DNA from supernatants of Triton X-100 lysates was prepared for analysis of fragmentation, as described previously in detail (32, 33). DNA was separated by electrophoresis through a 1% agarose gel, and the DNA fragments were visualized by UV light after being stained with ethidium bromide. Controls included DNA from PBS-treated cells cultured for 24 h, DNA from cells treated with 1000 ng/ml GMCSF for 24 h, DNA from cells irradiated with 2 Gy γ-rays 24 h prior to harvest, and DNA from cells preincubated for 2 h with 3000 ng/ml native recombinant GMCSF prior to treatment with 100 ng/ml DT_{ct}GMCSF for 24 h.

RESULTS

DT_{ct}GMCSF-induced Apoptosis in GMCSF Receptor-positive Human AML Cells. DT_{ct}GMCSF contains both the catalytic and transmembrane translocation domains of DT linked via a short, sterically neutral peptide incorporated as a spacer to GMCSF, replacing the DT receptor binding domain (Fig. 1*A*). HL-60 is a p53-deficient myeloid leukemia cell line expressing 77 ± 12 high-affinity GMCSF-R/cell with a K_d of 34.8 ± 3.8 pM (24). As shown in Fig. 1*B*, DT_{ct}GMCSF exhibited high-affinity binding to the GMCSF-R on HL-60 cells and was able to displace ^{125}I-labeled GMCSF from HL-60 myeloid leukemia cells with an EC_{50} value (*i.e.*, concentration that effected 50% displacement of ^{125}I-GMCSF from the GMCSF-R) of 0.199 nM. DT_{ct}GMCSF effectively inhibited





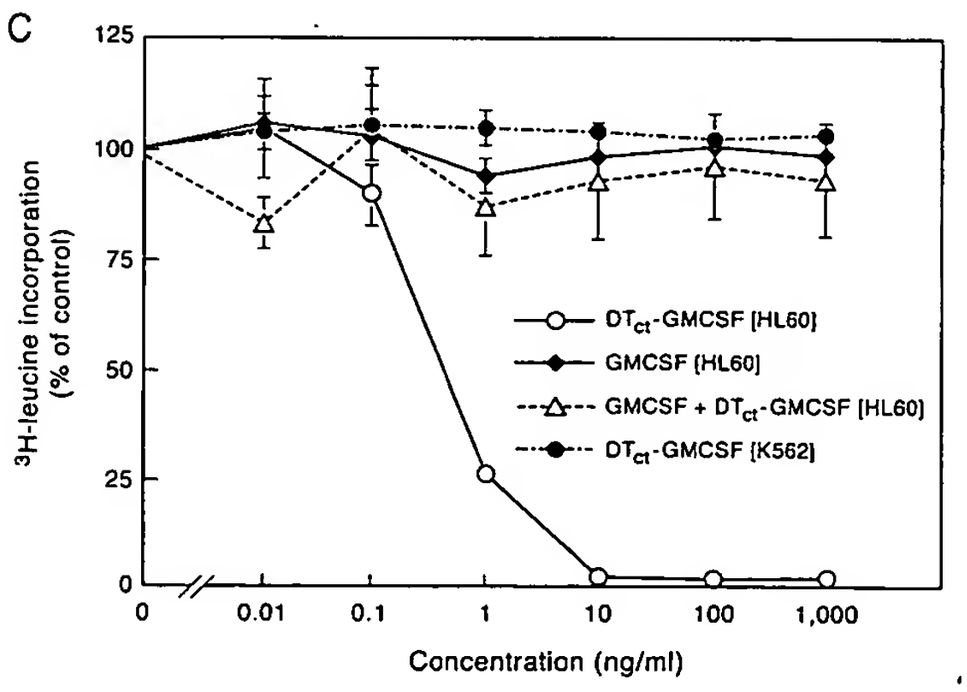


Fig. 1 A GMCSF-R-specific recombinant fusion toxin. *A*, $DT_{ct}GMCSF$ fusion toxin structure. The amino-terminal 385 residues of native DT containing the ADP-ribosyltransferase catalytic (C) and membrane translocation (T) domains are preserved in $DT_{ct}GMCSF$. The recombinant fusion toxin incorporates a short linking sequence [L: Ser-$(Gly)_4$-Ser-Met] and human GMCSF at the carboxyl terminus, replacing the native receptor binding domain of DT. *B*, comparison of binding activity of $DT_{ct}GMCSF$ with native GMCSF. Increasing concentrations of the fusion toxin or native growth factor were incubated at 4°C for 2 h with 40 pM ^{125}I-GMCSF and HL-60 myeloid leukemia cells (4×10^6 cells in 400-μl aliquots of IMDM binding buffer). *Bars*, SD of triplicate samples; not displayed when less than the size of the data point in the figure. *C*, $DT_{ct}GMCSF$-induced inhibition of protein synthesis. Cell lines were incubated with increasing concentrations of $DT_{ct}GMCSF$ or GMCSF for 24 h at 37°C. For competition studies, cells were preincubated with 1000 ng/ml GMCSF for 1 h prior to the addition of $DT_{ct}GMCSF$. Protein synthesis inhibition was calculated after a 2-h pulse of [^{3}H]leucine and measurement of TCA-precipitable radioactivity with comparison to controls treated with PBS alone.

protein synthesis in HL-60 cells with an IC_{50} of approximately 0.5 ng/ml (~9 pM; Fig. 1*C*). Native human GMCSF did not inhibit protein synthesis in HL-60 cells, and the protein synthesis-inhibitory activity of $DT_{ct}GMCSF$ could be abolished by preincubation of cells with excess native human GMCSF, which competes for binding to the GMCSF-R. In contrast, protein synthesis in the erythroleukemia cell line K562, which does not express the high-affinity GMCSF-R (34), was not affected by $DT_{ct}GMCSF$ concentrations as high as 1000 ng/ml (~17 nM). Thus, the observed inhibition of protein synthesis in $DT_{ct}GMCSF$-treated HL-60 cells was mediated through specific binding of this fusion toxin to the high-affinity GMCSF-R.

Because the final pathway for cell death after intoxication with $DT_{ct}GMCSF$ is unknown, we investigated whether the inhibition of protein synthesis effected by the $DT_{ct}GMCSF$-specific ADP-ribosylation of the diphthamide site of elongation factor 2 could subsequently trigger apoptosis in GMCSF-R-positive human leukemia cells. The failure of normal apoptosis pathways or resistance to chemotherapy-induced apoptosis is an important mechanism underpinning the biology of chemotherapy-refractory leukemias. Several studies have demonstrated that inhibitors of protein synthesis, including DT and cycloheximide, activate apoptotic cell death programs in a variety of leukemia cell lines (35, 36). We tested the cytotoxicity of $DT_{ct}GMCSF$ against HL-60 cells deficient in p53 because mutation of the *p53* gene and loss of its functional tumor suppressor activity is frequently observed in aggressive malignancies and is associated with a failure of the induction of apoptotic cell death and a poor response to conventional therapies that damage DNA (37, 38). Within 4 h of exposure to 100 ng/ml of $DT_{ct}GMCSF$, approximately 50–75% of HL-60 cells showed morphological changes consistent with extensive apoptotic damage, including pronounced shrinkage of the cells, nuclear chromatin condensation, segmentation of the nucleus, and plasma membrane blebbing (Fig. 2). In contrast, native human GMCSF did not induce apoptosis in HL-60 cells (Fig. 2*B*). A 24-h exposure to 100 or 1000 ng/ml $DT_{ct}GMCSF$ was more effective than a 4-h exposure; after 24 h of treatment, very few cells remained detectable, and virtually all of these cells showed signs of extensive damage consistent with apoptosis (Fig. 2). A few isolated cells showed distinctive features of necrosis, including swelling of the nucleus and cytoplasm and loss of nuclear and cytoplasmic basophilia (Fig. 2*E*).

Examination of DNA from $DT_{ct}GMCSF$-treated (but not GMCSF-treated) HL-60 cells on agarose gels revealed a dose-dependent and ladder-like fragmentation pattern consistent with endonucleolytic cleavage of the DNA into oligonucleosome-length fragments during apoptotic cell death (Fig. 3). $DT_{ct}GMCSF$ did not cause DNA fragmentation in the GMCSF-R-negative NALM-6 pre-B lineage leukemia cell line, and preincubation of the HL-60 cells with excess native GMCSF was able to prevent DNA fragmentation (Fig. 3, *A* and *B*). Thus, $DT_{ct}GMCSF$-induced apoptosis was mediated by the GMCSF-R-specific binding of the fusion toxin to leukemia cells.

$DT_{ct}GMCSF$-induced Apoptosis in Multidrug-resistant and Radiation-resistant Human AML Cells. We next studied the antileukemic activity of $DT_{ct}GMCSF$ against HL-60/VCR cells, which express a P-glycoprotein associated MDR phenotype, and HL-60/ADR cells, which express a MRP-

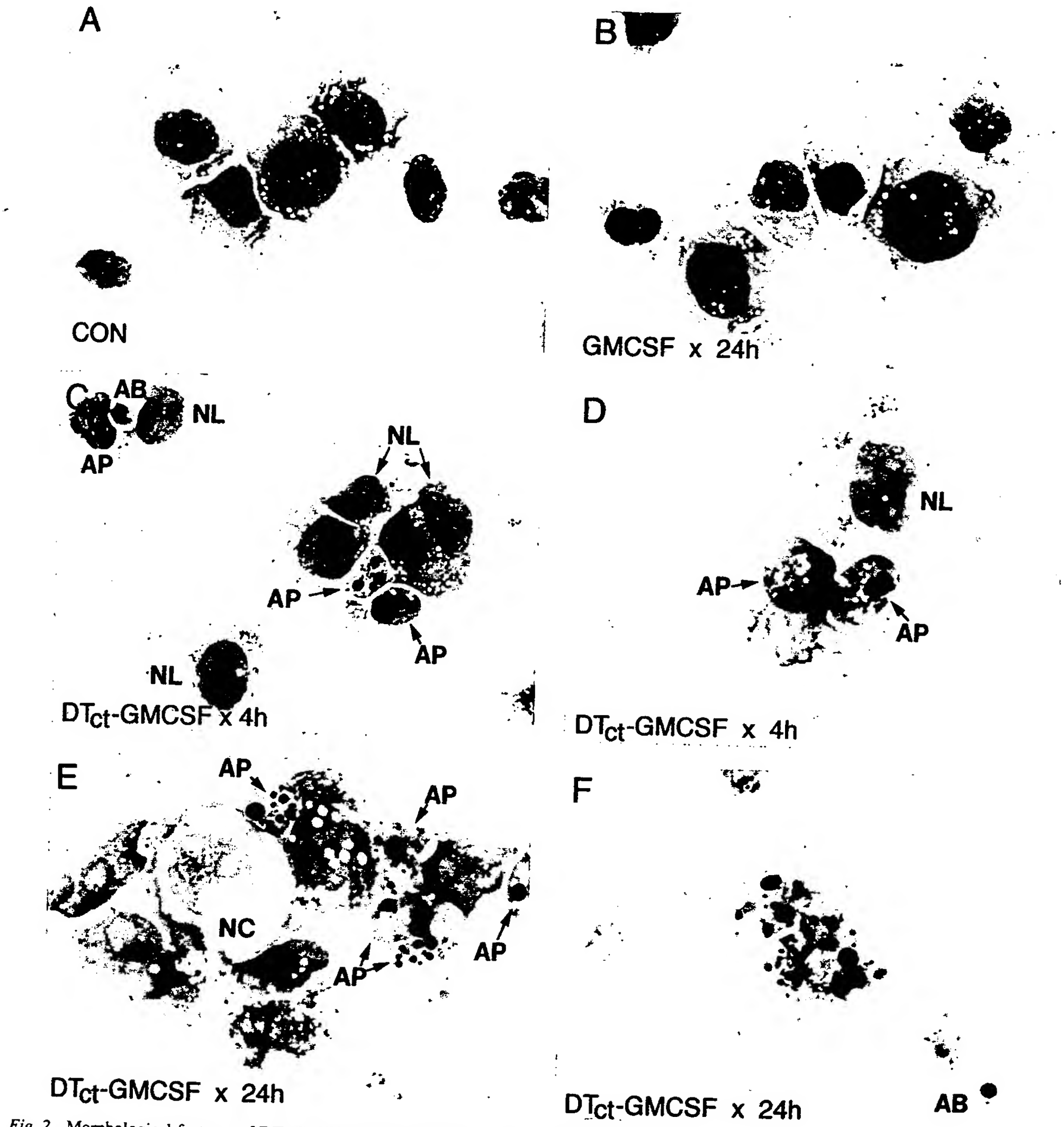


Fig. 2 Morphological features of DT_{ct}GMCSF-treated leukemia cells undergoing apoptosis. After treatment with 100 ng/ml DT_{ct}GMCSF for 4 or 24 h, HL-60 cells were examined on Wright-Giemsa-stained cytospin slides for morphological changes characteristic of apoptosis, including nuclear chromatin condensation, segmentation of the nucleus, and plasma membrane blebs. Apoptotic cells (*AP*) and apoptotic bodies (*AB*), necrotic cells (NC) as well as morphologically healthy cells (*NL*), are indicated by *arrows*. Control cells were treated for 24 h with PBS (*CON*) or 100 ng/ml native recombinant GMCSF.

associated MDR phenotype. On agarose gels, DNA from DT_{ct}GMCSF-treated HL-60/VCR and HL-60/ADR myeloid leukemia cells exhibited a ladder-like fragmentation pattern consistent with apoptosis (Fig. 3*C*). Thus, MDR due to overexpression of P-glycoprotein or MRP does not protect GMCSF-R-positive AML cells from DT_{ct}GMCSF-induced apoptosis.

We also studied the ability of DT_{ct}GMCSF to induce apoptosis in primary leukemic cells from four patients with chemotherapy-refractory relapsed AML. In one case (case 1), DT_{ct}GMCSF was as effective as 2 Gy γ-rays in inducing apoptosis, and in three additional cases (cases 2, 3, and 4), DT_{ct}GMCSF induced DNA fragmentation, whereas 2 Gy γ-rays

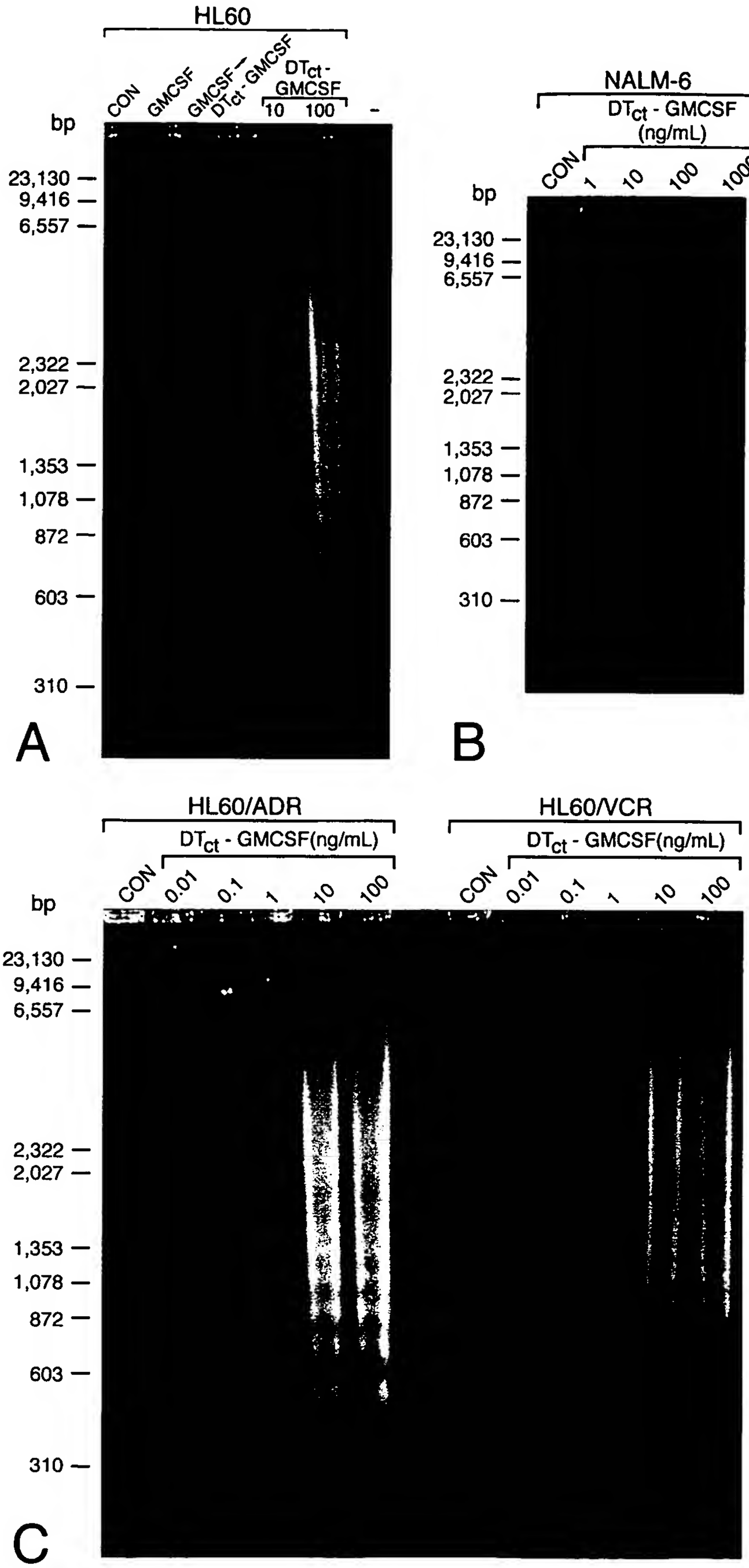


Fig. 3 Analysis of $DT_{ct}GMCSF$ dose-dependent induction of internucleosomal DNA fragmentation in GMCSF-R-positive leukemia cells. Cells were harvested 24 h after continuous exposure to the $DT_{ct}GMCSF$ fusion toxin, and DNA from supernatants of Triton X-100 lysates was prepared for analysis of fragmentation. DNA was separated by electrophoresis through a 1% agarose gel, and the DNA fragments were visualized by UV light after staining with ethidium bromide. Controls included DNA from PBS-treated cells cultured for 24 h (*CON*), DNA from cells treated with 1000 ng/ml GMCSF for 24 h (*GMCSF*), and DNA from cells preincubated for 2 h with 3000 ng/ml GMCSF prior to treatment with 100 ng/ml $DT_{ct}GMCSF$ for 24 h (*GMCSF* → *DT_{ct}GMCSF*). *A*, p53-deficient HL-60 myeloid leukemia cells bearing high-affinity GMCSF-R. *B*, GMCSF-R-negative NALM-6 pre-B leukemia cells. *C*, multidrug-resistant HL-60/VCR leukemia cells expressing high levels of Pgp and HL-60/ADR leukemia cells expressing MRP treated with $DT_{ct}GMCSF$. Molecular size markers (in bp) are indicated at the *left* in each panel.

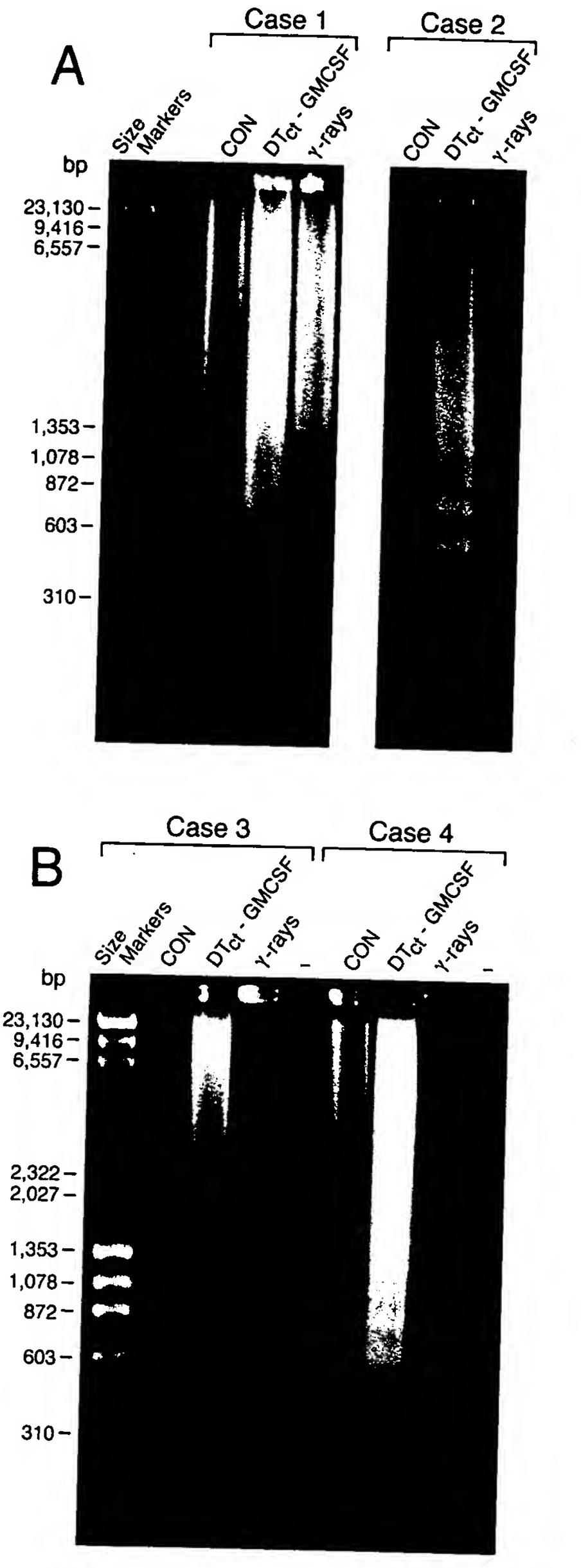


Fig. 4 $DT_{ct}GMCSF$ induces apoptosis in primary leukemic cells from relapsed AML patients. Bone marrow-derived leukemic cells from 4 therapy-refractory AML patients in relapse were harvested 24 h after continuous exposure to 100 ng/ml $DT_{ct}GMCSF$ fusion toxin or PBS (*CON*), or irradiation with 2 Gy γ-rays, and DNA from Triton X-100 lysates was prepared for analysis of fragmentation. Molecular size markers (in bp) are indicated at the *left* of each panel.

did not (Fig. 4). Thus, $DT_{ct}GMCSF$ caused apoptosis of primary leukemic cells from relapsed AML patients regardless of their radiation sensitivity.

DISCUSSION

The emergence of chemotherapy- and radiation-resistant clones of leukemic cells is the major obstacle to the cure of AML and is caused by several mechanisms. The multidrug resistance phenotype has been implicated as one such major cause for treatment failure in AML and results from overexpression of transmembrane transporter molecules exporting a variety of natural cytotoxic agents with different mechanisms of action (39, 40). The MDR phenotype in AML can be artificially produced by the overexpression of Pgp (also known as P-170; Refs. 41 and 42), and the MRP (29, 43, 44). Expression of Pgp in primary leukemic cells from AML patients has been associated with higher rates of chemotherapy remission induction failures, shorter relapse-free intervals, and increased rates of relapse (45–50). In addition, increased MRP expression has also been identified in the leukemic cells of a significant proportion of patients with relapsed AML (51, 52). Attempts to overcome drug resistance in AML by increasing chemotherapy regimen dose intensity, including myeloablative chemotherapy followed by allogeneic or autologous bone marrow transplantation, or by the use of agents that inhibit the activity of MDR-associated transporter molecules, have been associated with considerable morbidity and mortality, and have effected only modest improvements in the overall survival of AML patients (4–6). The utility of dose-intensive therapy in the management of therapy-resistant malignancies is severely limited because of the toxicities inflicted upon normal tissues.

Paralleling the contribution of the MDR phenotype to the poor survival of AML patients are the observations that clinically aggressive and drug-resistant forms of AML are associated with the autonomous growth of leukemic blasts related to the activation of hematopoietic growth factor-related proliferative pathways (53, 54). In particular, GMCSF and its receptor appear to play a prominent role in AML leukemogenesis, and large subsets of patients may possess leukemic cells with direct activation of GMCSF-related growth loops (55–57) or indirect activation of GMCSF growth loops caused by interleukin 1 or other cytokines (58–60). The role of GMCSF in the etiology and maintenance of AML is underscored by the observation that autonomous proliferation of AML blasts in culture can be abrogated in over 80% of patient samples by exposure to either a neutralizing anti-GMCSF antibody or an antisense oligonucleotide directed against the GMCSF transcript (61). Moreover, there is substantial evidence that the leukemic blasts from the majority of patients with AML express GMCSF-R (62–64), and GMCSF-specific binding can be demonstrated to leukemic cells from virtually all AML samples surveyed when refined binding studies are used (65). The observation that GMCSF and its receptor may function prominently in the etiology and maintenance of AML has prompted attempts to use it as a "priming agent" to stimulate the transit of leukemic cells into S phase and thereby increase sensitivity to cytotoxic chemotherapies (66–71). Although this approach is intriguing, it does not provide a vehicle to overcome important AML therapy resistance resulting from MDR-associated mechanisms or resistance to apoptotic cell death.

A novel strategy for tumor-specific biotherapy has been developed by using genetic engineering to target protein synthesis inhibiting toxins, including DT (14–19) and *Pseudomonas* exotoxin A (72, 73), to growth factor receptors found on neoplastic cells. Notably, it has been observed previously that *Pseudomonas* exotoxin A can effectively kill drug-resistant cancer cells that express the Pgp (74). We have used genetic engineering to create a novel fusion toxin, $DT_{ct}GMCSF$, to redirect the lethal action of DT to the high-affinity GMCSF-R found on AML cells. We postulated that $DT_{ct}GMCSF$ would be an active agent against drug-resistant leukemia cells possessing the MDR phenotype or deficient in p53 expression because (*a*) it gains entry to target cells by a unique mechanism (GMCSF-R binding); (*b*) it kills cells by a mechanism of action (protein synthesis inhibition) that is distinct from other chemotherapeutic agents and radiation; and (*c*) it is not apparently similar in structure to other Pgp or MRP substrates. Our results presented in this report demonstrate that $DT_{ct}GMCSF$ is able to overcome classical mechanisms of drug and radiation resistance, as well as circumvent the failure to engage apoptotic pathways. The recombinant fusion toxin $DT_{ct}GMCSF$ may thus serve as an effective treatment for AML.

Resistance to the induction of the programmed cell death mechanism after malignant cells have incurred damage from chemotherapy or radiation is another important mechanism of treatment resistance in AML and other cancers (75). Known also by the descriptive term apoptosis, this specific cell death mechanism is characterized by distinct morphological and ultrastructural features and by endonuclease-mediated cleavage of DNA into oligonucleosome-length fragments (76–78). Ionizing radiation and chemotherapeutic drugs have been shown to inflict cellular damage that engage cellular mechanisms, likely including the interleukin-1β-converting enzyme-related protease family, that result in the apoptotic cell death of cancer cells (79–81). The p53 tumor suppressor protein appears to play a major role in induction of apoptosis (37, 38). Cancers with deficient function of p53 are thus postulated to be therapy resistant (82), and treatment resistance and relapse have been associated with inactivation of p53 and defective induction of apoptosis in immunocompromised mouse tumor models (83). Human hematological malignancies bearing *p53* gene mutations exhibit *in vitro* resistance to DNA-damaging agents, including chemotherapy and radiation (84), and are also associated with a poor response to therapy and short survival (85, 86). In our studies, treatment of p53-deficient HL-60 myeloid leukemia cells with $DT_{ct}GMCSF$ resulted in the rapid and efficient induction of apoptosis, suggesting that the fusion toxin could potentially circumvent chemotherapy and radiation resistance resulting from p53-deficiency and failure of apoptosis. The $DT_{ct}GMCSF$-induced DNA fragmentation and apoptotic alterations in morphology in HL-60 leukemia cells were not associated with evidence of terminal maturation, nor were they associated with growth factor deprivation, and these cells are GMCSF independent. $DT_{ct}GMCSF$-induced DNA fragmentation was most likely a consequence of the DT catalytic domain-induced inhibition of protein synthesis because native recombinant GMCSF did not cause DNA fragmentation, and a variety of biochemical and genetic studies have demonstrated that the DT catalytic domain does not possess direct DNase activity (87–89). These observations suggest that, at least in some leukemias, the induction of apoptosis by $DT_{ct}GMCSF$-mediated inhibition of protein synthesis involves p53-independent pathways. The precise pathway of apoptosis induction is not yet defined, and we are examining whether cells with defects in the downstream effectors of apoptotic cell death (*e.g.*, the interleukin-1β-converting enzyme protease pathways) remain susceptible to the cytotoxic consequences of treatment with $DT_{ct}GMCSF$.

We further postulated that $DT_{ct}GMCSF$ would be an active agent against leukemia cells possessing the MDR phenotype because its structure is distinct from other P-glycoprotein or MRP substrates and it kills target cells by a unique mechanism of action. We found that overexpression of P-glycoprotein or MRP does not appear to cause a significant decrease in $DT_{ct}GMCSF$ cytotoxicity in human myeloid leukemia cells and that $DT_{ct}GMCSF$ efficiently induces apoptosis in highly drug-resistant cells. The prominent role of the MDR phenotype in causing treatment failure and ultimate mortality in AML suggests that $DT_{ct}GMCSF$ may be useful to complement the activity of conventional antineoplastic agents in the treatment of AML.

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A Recombinant Fusion Toxin Targeted to the Granulocyte-Macrophage Colony-Stimulating Factor Receptor

ANNE E. BENDEL, YU SHAO, STELLA M. DAVIES, BERTA WARMAN, CHANG H. YANG, KEVIN G. WADDICK, FATIH M. UCKUN and JOHN P. PERENTESIS*,†

Biotherapy Institute and Departments of Pediatrics, Therapeutic Radiology-Radiation Oncology, Biochemistry, Pharmacology, University of Minnesota Academic Health Center, Minneapolis, Minnesota, USA

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Human granulocyte-macrophage colony stimulating factor (GMCSF) and its high affinity receptor function to regulate the proliferation and differentiation of myeloid lineage hematopoietic cells, and may participate in the pathogenesis of many malignant myeloid diseases. We have used genetic engineering based on the elucidated molecular structures of human granulocyte-macrophage colony-stimulating factor and diphtheria toxin (DT) to produce a recombinant fusion toxin, DT_{ct}GMCSF, that targets diphtheria toxin to high affinity GMCSF receptors expressed on the surface of blast cells from a large fraction of patients with acute myeloid leukemia (AML). DT_{ct}GMCSF was specifically immunoreactive with anti-diphtheria toxin and anti-GMCSF antiseras, and exhibited the characteristic catalytic activity of diphtheria toxin, catalyzing the *in vitro* ADP-ribosylation of purified elongation factor 2. The cytotoxic effects of DT_{ct}GMCSF were examined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-tetrazolium (MTT) bromide assay of cell viability and *in vivo* assays of protein synthesis inhibition. DT_{ct}GMCSF were specifically cytotoxic to human leukemia cell lines bearing high affinity receptors for human GMCSF with IC_{50} of 10^{-9} to 10^{-11}M. It was not toxic to mammalian hematopoietic cell lines lacking human GMCSF (hGMCSF) receptors. In receptor positive cells, cytotoxicity can be specifically blocked by a large excess of hGMCSF, confirming that its cytotoxicity is mediated through the hGMCSF receptor. Though DT_{ct}GMCSF inhibited granulocyte-macrophage colony formation by committed myeloid progenitor cells (CFU-GM), it did not significantly affect erythroid burst formation by committed erythroid progenitor cells (BFU-E), or mixed granulocyte-erythroid-macrophage-megakaryocyte colony formation by pluripotent multilineage progenitor cells (CFU-GEMM). DT_{ct}GMCSF holds promise for the treatment of myeloid lineage malignancies, and is a useful reagent to study hematopoiesis.

Keywords: Myeloid leukemia, diphtheria toxin–GM-CSF fusion toxin, GM-CSF receptor

*Corresponding author. Tel.: (612) 627-1920. Fax: (612) 627-1928.
†Present address: University of Minnesota, Biotherapy Institute, 2625 Patton Road, Roseville, MN 55113.

INTRODUCTION

Acute myeloid leukemia (AML) is the most common form of acute leukemia in adults and the second most frequent leukemia in children.[1] Though the majority of patients with myeloid leukemias initially respond to chemotherapy, most will relapse and eventually succumb to their disease.[2] High dose intensity chemotherapy regimens have been used in an attempt to eradicate the drug-resistant clones that produce leukemia relapse, but these maneuvers have met with limited success. The safe administration of anticancer drugs to AML patients is limited by the damage that chemotherapy inflicts upon normal tissues. These nonspecific effects of conventional intensive chemotherapy are responsible for the severe side effects of the cancer treatments, and the limitations in effective drug dosaging resulting in the regrowth of leukemia.

Recent studies have provided experimental evidence that cell-type specific biotherapeutics hold promise for the treatment of refractory human cancer.[3–6] We therefore focused our efforts on designing a biotherapeutic strategy against AML that allows the targeted delivery of a highly potent cytotoxic substance to myeloid leukemia cells. To identify an appropriate leukemic cell surface target for directed biotherapy, we capitalized upon the observations that implicated the dysregulation of expression of cytokines including granulocyte-macrophage colony stimulating factor (GMCSF) or its high affinity receptor, in AML leukemogenesis.[7–10] GMCSF has been observed to be the major growth factor responsible for the direct autonomous growth of AML blasts in culture,[11,12] and leukemic cells from a majority of patients with AML exhibit an autonomous growth pattern related to GMCSF autocrine or paracrine production and secretion.[13] The autonomous growth of leukemic blasts related to growth factor proliferative pathways has been associated with aggressive and drug-resistant forms of AML, and a reduction in survival.[14,15] Additional patients may express functional GMCSF receptors on their blasts without direct involvement of pathological growth stimulatory loops in leukemogenesis.

Diphtheria toxin (DT) is one of the most toxic substances found in nature, and human cells can be killed as a consequence of the catalytic inactivation of protein synthesis resulting from the entry of a single molecule of the toxin into the cytoplasm.[16] DT-mediated protein synthesis inhibition may provide a particularly efficient and complementary mechanism for cell killing when compared to other anticancer agents employed for current AML therapies. Molecular genetic, biochemical, and X-ray crystallographic analyses have revealed that DT possesses functionally distinct structural domains corresponding to (a) an ADP-ribosyltransferase catalytic activity ("C" domain), (b) a membrane translocation activity ("T" domain), and (c) a unique receptor binding moiety ("R" domain) (reviewed in reference 17). We have used genetic engineering based upon the solved crystal structure of DT[18,19] to create a novel fusion toxin, DT_{ct}GMCSF, to redirect the lethal action of DT to the high affinity GMCSF-R found on AML blasts. In DT_{ct}GMCSF, the catalytic ("C") and translocation ("T") domains of DT are preserved, but the native receptor-binding domain of DT, which mediates its indiscriminate binding to human cells, is genetically replaced with human GMCSF.

Murphy and coworkers have successfully employed genetic engineering to redirect the lethal action of diphtheria toxin to effectively target *in vivo* human malignancies expressing high affinity cytokine and growth factor receptors found on neoplastic cells.[20–23] Early clinical trials of their recombinant diphtheria toxin-interleukin 2 fusion toxin (DAB_{486}IL-2) have shown that it is well tolerated and displays significant activity in patients with chemotherapy refractory hematologic malignancies expressing the IL-2 receptor.[24,25] DAB_{486}IL-2 specifically binds only to cells that express the high affinity receptor for IL-2 with the induction of rapid receptor-mediated endocytosis and consequent cell killing by protein synthesis inhibition,[26,27] and this may account for its favorable toxicity profile. The diphtheria toxin—IL-2 recombinant fusion toxin has been refined to reduce molecular size and susceptibility to proteolysis. This new fusion protein, DAB_{389}IL-2, appears to possess greater potency than its predecessor in pre-clinical trials and is cur-

rently in Phase I clinical trials.[28] The scientific basis of the clinical efficacy and toxicity profiles of $DAB_{486}IL$-2 and $DAB_{389}IL$-2 may serve as a paradigm for the use of other recombinant fusion toxins such as $DT_{ct}GMCSF$ that also enter cells after binding to multimeric high-affinity receptor complexes.

In this study, we examined the ability of DT_{ct} GMCSF to effect specific cytotoxicity to leukemic blasts expressing GMCSF receptors. $DT_{ct}GMCSF$ is specifically toxic to myeloid leukemia cell lines bearing the high-affinity hGMCSF receptor but has no effect on other hematopoietic cell lines that lack these receptors. These cytotoxic effects of $DT_{ct}GMCSF$ can be blocked by antisera to diphtheria toxin or with a large excess of hGMCSF, confirming that the cytotoxicity is mediated through the hGMCSF receptor. We also examined the effects of $DT_{ct}GMCSF$ on the growth of committed bone marrow progenitors. We plan to develop $DT_{ct}GMCSF$ as a reagent to define subsets of myeloid leukemia which may respond to therapy targeted at the GMCSF receptor, and as a potential adjuvant to conventional chemotherapies for myeloid leukemias.

MATERIALS AND METHODS

Nucleic Acids and DNA Cloning The polymerase chain reaction (PCR) was employed for mutagenesis of the diphtheria toxin gene to delete the coding region for the native toxin binding domain, and provide coding sequences for a translation initiation ATG codon, a seven residue linker segment for fusion with the GMCSF gene, and convenient flanking NcoI restriction enzyme sites for cloning. PCR mutagenesis primers included a 5′ primer (5′-GCCATGGGCGCTGATGATGTTGTTGATTC-3′) introducing an NcoI restriction enzyme site and ATG codon, and a 3′ primer (5′-GCCATGGAGCCACCTCCACCCGATTTATGCCCCGGAGAATACGC-3′) incorporating sequences encoding a linker domain for steric spacing of the GMCSF gene and an NcoI restriction enzyme site. The expression plasmid $pET11dDT_{ct}GMCSF$ was constructed by the cloning of the intact DAB Nco I gene cassette into the Nco I site of pET11d-GMCSF as shown in Figure 1. Cloning strategies and other genetic manipulations were positioned to assure maintenance of the translational reading frame, and fidelity of PCR amplification and genetic constructions were confirmed by DNA sequencing. Oligonucleotide primers were synthesized with an Applied Biosystems 394 DNA synthesizer at the University of Minnesota Microchemical Facility. A synthetic cDNA encoding human GMCSF using E. coli codon preferences was obtained from R & D Systems (Minneapolis, MN). The plasmid containing the diphtheria toxin gene has been described previously.[29] Plasmid DNAs were prepared by either the alkaline lysis method with purification on cesium chloride/ethidium bromide gradients, or by use of the Wizard DNA purification resin (Promega, Madison, WI). DNA fragments amplified by the polymerase chain reaction (PCR) were initially cloned into the pT7Blue vector as directed by the manufacturer (Novagen), with DNA sequencing confirmation by the dideoxy method of Sanger using CircumVent thermal cycling reagents (New England Biolabs, Beverly, MA). Restriction endonucleases. Taq DNA polymerase, and T4 DNA ligase were procured from BRL-Life Technologies (Gaithersburg, MD). Promega, New England Biolabs, or Perkin Elmer (Norwalk, CT), and used according to the specifications directed by the manufacturer. Standard techniques were employed for other manipulations of DNA including agarose gel electrophoresis, isolation and purification of restriction endonuclease fragments, cloning, and plasmid transformation into bacteria.[30]

Expression and Purification of the Recombinant Fusion Toxin $DT_{ct}GMCSF$ All manipulations of E. coli bearing intact recombinant fusion toxin were performed under modified Biosafety Level 3 (BL3) containment practices. E. coli HMS174(de3)plysS transformed with $pET11d\text{-}DT_{ct}GMCSF$ was grown at 37°C in LB medium with carbenicillin (50 μg/ml) to an absorbance (Å600) of 0.4–0.6. Expression of the fusion gene was induced by the addition of isopropyl-B-D-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM. The bacterial cells were collected by centrifugation after

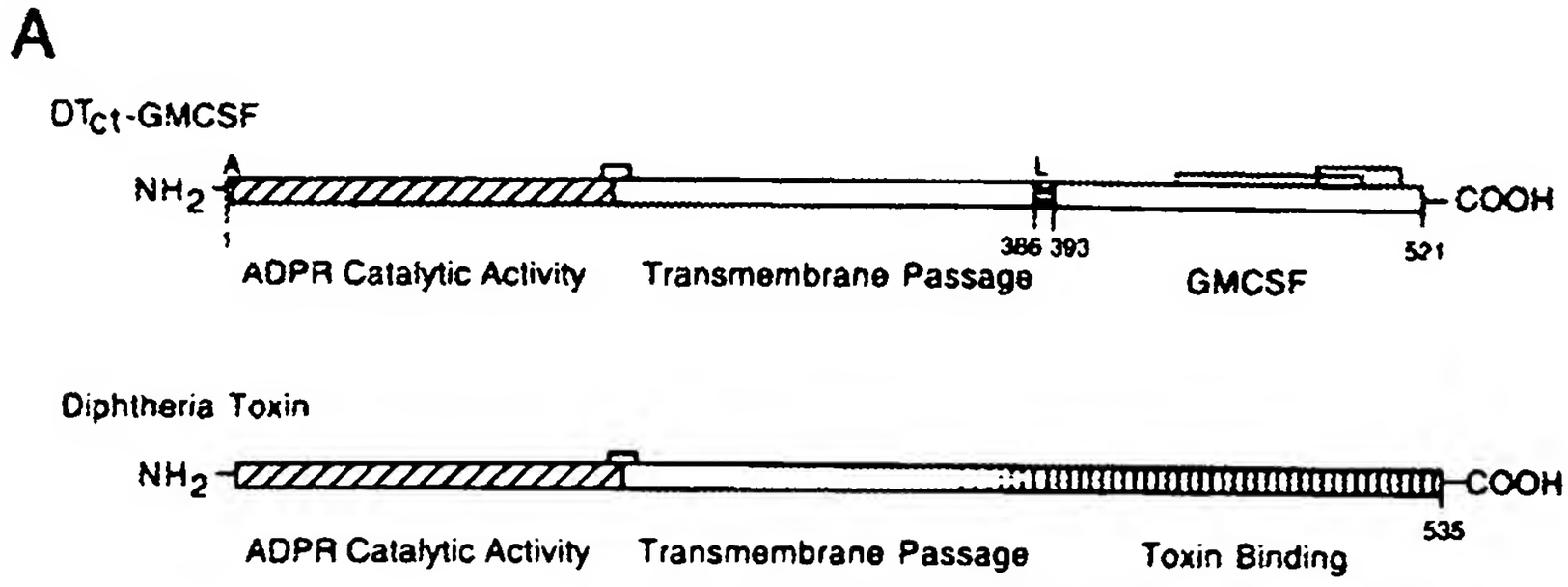


FIGURE 1 (A) Structure of the recombinant $DT_{ct}GMCSF$ fusion toxin and comparision to diphtheria toxin. The native receptor-binding domain of diphtheria toxin was genetically deleted and replaced with human GMCSF, separated by a short linking sequence ("L": Ser-$(Gly)_4$-Ser-Met). The genetic addition of an ATG codon was used to introduce a methionine residue to the amino terminus of the fusion toxin ("A" = Met). (B) Construction of the recombinant growth factor—toxin fusion expression vector pET11d-DT_{ct}GMCSF. A synthetic cDNA encoding mature human GMCSF was cloned between the Nco I and Bam HI sites of plasmid pET11d downstream of the T7 promoter to produce pET11d-GMCSF. PCR mutagenesis of the diphtheria toxin gene was employed to obtain a NcoI DAB gene cassette that included: (*i*) the addition of an ATG methionine translation initiation codon immediately 5′ of the initial GGC glycine codon of mature native diphtheria toxin, (*ii*) a short 3′ linker sequence encoding seven amino acid [Ser-$(Gly)_4$-Ser-Met] residues downstream of diphtheria toxin lysine residue 385, and (*iii*) flanking Nco I restriction endonuclease sites. Expression plasmid pET11dDT_{ct}GMCSF was constructed by the cloning of the intact DAB Nco I gene cassette into the Nco I site of pET11d-GMCSF.

one hour of induction, and resuspended in 50 mM potassium phosphate, 10 mM EDTA, 750 mM NaCl, 0.1% Tween 20, pH 8.0. Lysis of the cells was achieved by freezing in a dry ice/ethanol bath followed by thawing and sonication. The soluble extract was filtered through a 0.2 μm filter (Millipore, Bedford, MA) and applied to a 4 ml anti-diphtheria toxin immunoaffinity column. The immunoaffinity column was prepared with Affinica Antibody Orientation Kit (Schleicher and Schuell, Keene, NH) using equine diphtheria antitoxin. Bound proteins were subsequently eluted from the immunoaffinity column with 4 M guanidine hydrochloride, 100 mM potassium phosphate, 0.1% Tween 20, pH 7.2. Eluate fractions containing the fusion toxin were dialyzed exhaustively in 20 mM Tris, 150 mM NaCl, pH 7.5 with 5 buffer changes over 18 hrs using 12kD exclusion dialysis tubing. The dialyzed fusion toxin was applied onto a Mono Q HR 5/5 chromatography column (Pharmacia, Piscataway, NJ) and eluted at a flow rate of 1 ml/min over 30 min with a linear gradient of NaCl (150–500mM in 20mM Tris, pH 7.5). The purified fusion toxin was concentrated 10–20 fold in a 30 kD exclusion filter (Amicon) followed by exhaustive dialysis in 2 L PBS with 5 buffer changes over 18 hrs. Protein concentration was determined by Bio-Rad Protein Assay. Over 100 μg of purified $DT_{cl}GMCSF$ protein was consistently obtained from 1 liter pilot cultures.

Biochemical Characterization of Recombinant $DT_{cl}GMCSF$ Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analyses using goat anti-human GMCSF (R & D Systems) or equine diphtheria antitoxin (anti-DT; Connaught) antibodies were performed by standard methods[29] using 10–15% gels in a Mini-Protean II gel apparatus (Bio-Rad). Diphtheria toxin and human GMCSF standards were obtained from Connaught Laboratories and R & D Systems (Minneapolis, MN), respectively. Primary antibodies were used at a dilution of 1:5000. Secondary antibodies, goat anti-horse (Bethyl laboratories) and rabbit anti-goat (Calbiochem), covalently linked to horseradish peroxidase were used at a 1:10,000 dilution. The ADP-ribosyltransferase catalytic activity of the recombinant toxin was determined by measuring incorporation into purified *Saccharomyces cerevisiae* EF-2 or into the EF-2 of reticulocyte lysates. Approximately 300 ng of $DT_{cl}GMCSF$ or 200 ng of nicked diphtheria toxin was incubated at 37°C with 10 μg of EF-2 in a reaction mixture containing 20 mM Tris-HCl (pH 7.5), 50 mM dithiothreitol, 1 mM EDTA, and 2.5 μM [adenylate-^{32}P]NAD$^+$ (≈1 × 10^6 cpm) and analyzed for ADP-ribose incorporation.[31]

Cell Lines and Culture Conditions TF-1, a GMCSF-dependent human early myeloid/erythroleukemia cell line,[32] was provided by Dr. J. Winkleman (University of Cincinnati, Cincinnati, OH). Other GMCSF-R—bearing human leukemia cell lines included the human acute promyelocytic leukemia cell line HL-60,[33] the human monocytic leukemia cell line THP-1,[34] the mixed lineage leukemia cell line MV4-11,[35] and the highly radiation-resistant mixed lineage acute leukemia cell line RS4;11[36] expressing high levels of Bcl-2 protein.[3] These cell lines as well as the GMCSF-R—negative control leukemia cell line K562[37] and pre-B leukemia cell line NALM-6[38] were obtained from the American Type Culture Collection (Rockville, MD). The murine leukemia cell line NFS-60[39] was the gift of Dr. J. Ihle (St. Jude Children's Research Hospital, Memphis, TN). TF-1 cells were maintained in RPMI, 10% FBS, 50 U/ml penicillin, 50 μg/ml streptomycin, 5 ng/ml human GMCSF. NFS-60 cells were maintained in RPMI, 10% FBS, 50 μ/ml penicillin, 50 μg/ml streptomycin, and 0.5 ng/ml human GCSF. HL-60 and MV4-11 cells were maintained in IMDM, 10% FBS and 50 U/ml penicillin, and 50 μg/ml streptomycin. THP-1 and K562 cells were maintained in RPMI, 10% FBS, 50 U/ml penicillin, 50 μg/ml streptomycin, and 5 × 10^{-5}M 2-mercaptoethanol (for THP-1 cells only).

Cytotoxicity Assays For protein synthesis inhibition assays 1 × 10^5 cells per well were seeded into 96 well V-bottom plates containing leucine-free Roswell Park Memorial Institute (RPMI) medium prior to the addition of DT or $DT_{cl}GMCSF$ and incubated at 37°C for 48 hours. MEM ^{3}H-leucine (L-[4,5-^{3}H]) (DuPont-NEN) was added to a final concentration of 1 μCi/well with a four hour pulse incubation. Cells were lysed with 4M KOH and the insoluble protein was precipi-

tated with 40% trichloroacetic acid. A cell harvester (PH.D., Cambridge Technology Inc.) was used to collect the insoluble protein on glass fiber filters, and radioisotope incorporation into protein was measured in a Beckman LS7000 scintillation counter. Cytotoxicity was also measured by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assays.[40] For the MTT assays, cells were seeded into 96-well plates at a final concentration of 5×10^3 cells/well for TF-1, and $5–9 \times 10^4$ cells/well for HL-60, K562, NFS-60, MV4-11 and THP-1, and incubated at 37°C in a humidified incubator under a 5% CO_2 atmosphere for 16–24 hours. Dilutions of the $DT_{ct}GMCSF$ fusion toxin in PBS and 1% BSA or dilutions of vincristine in PBS/0.2% bovine serum albumin or doxorubicin in PBS/0.2% normal saline were added to each well and the incubation was continued for an additional 72–96 hours. MTT was added to a final concentration of 0.75 mg/ml with 4 hours of further incubation at 37°C. The dye was solubilized with 50% Isobutanol/10% SDS and cell viability was determined by measure of absorption (Å595) using a Bio-Rad Elisa Reader.

GMCSF Receptor Alpha Chain Expression Assay Individual leukemia cell lines were analyzed for GMCSF receptor alpha chain expression by the use of reverse-transcriptase PCR methodology to produce cellular cDNA followed by the use of gene-specific oligonucleotide primers for amplification and analysis of receptor expression. poly(A)$^+$ RNA was isolated directly from $\sim 10^5$ cells after detergent lysis with an oligo (dT) cellulose affinity resin using the Micro-FastTrack mRNA Isolation Kit (Invitrogen, San Diego, CA). Approximately 100 ng of poly(A)$^+$ RNA was used with random hexamer oligonucleotide primers and Moloney murine leukemia virus reverse transcriptase for first strand cDNA synthesis.[30] An internal primer set for the human GMCSF receptor alpha chain (5′ primer: 5′-AGAAATCGGATCTGCGAACAGTGGCACC-3′; 3′ primer: 5′-TCCAGGTACGACAGCTTCTGATAGGTCC-3′) obtained from Clontech Laboratories (Palo Alto, CA) was used to amplify a 682 base pair PCR fragment between nucleotides 69 and 749 of the receptor cDNA coding sequence. A series of control experiments were conducted with each set of PCR amplification experiments and included positive controls using human and murine beta-actin primer sets (Stratagene, La Jolla, CA) and negative controls lacking template DNA.

Clonogenic Leukemic and Normal Marrow Assays We used a highly sensitive in vitro limiting dilution clonogenic assay system[41,42] to determine the antileukemic efficacy of $DT_{ct}GMCSF$ against the clonogenic fraction of HL-60 myeloid leukemia cell line. Colony assays of normal bone marrow progenitor cells CFU-GEMM, CFU-GM, and BFU-E were previously described.[43] Bone marrow mononuclear cells from three healthy donors were kindly provided by Dr. J. McCullogh from the University of Minnesota Blood Bank. The percent control recovery values were calculated using the formula: % Control recovery = [Mean number of colony forming units in $DT_{ct}GMCSF$ treated samples/Mean number of colony forming units in untreated samples] × 100.

RESULTS

Design, Construction, and Expression of the Recombinant Growth Factor—Toxin Fusion DT_{ct} GMCSF The recombinant growth factor—toxin fusion expression vector pET11d-$DT_{ct}GMCSF$ was constructed in three major steps as depicted in Figure 1. An 392 bp NcoI—BamHI DNA fragment containing the coding sequence of mature human GMCSF was cloned between the Nco I and Bam HI sites of plasmid pET11d downstream of the T7 promoter to produce pET11d-GMCSF. PCR mutagenesis of the diphtheria toxin gene was employed to obtain an NcoI gene cassette that encoded 385 amino terminal residues of diphtheria toxin including the entire ADP-ribosyltransferase catalytic domain and the contiguous proximal portion of the toxin that is associated with translocation across cellular membranes (Fig 1A). The construction of this recombinant fusion toxin, $DT_{ct}GMCSF$, preserved the portion of the DT gene encoding amino acid residues 1-385 including

both the lethal catalytic ADP-ribosyltransferase and the transmembrane passage domains. This portion of the DT gene was fused to the gene that encodes the mature form of GMCSF, effectively deleting the native DT binding domain. In addition, a synthetic DNA sequence encoding a short Ser-$(Gly)_4$-Ser-Met intervening linker was inserted at the DT "hinge" site separating the DT and GMCSF moieties to ensure that the N-terminal helices of GMCSF would be available for participation in high-affinity receptor binding (Fig. 1B). High efficiency expression of $DT_{cl}GMCSF$ was achieved in E. coli followed by serial purification through anti-diphtheria toxin affinity chromatography, anion exchange chromatography and extensive dialysis.

SDS-polyacrylamide gel analysis of the uninduced and induced whole cell extract, immunoaffinity column eluate, and Mono Q chromatography column fractions revealed production and >95% purity of a monomeric protein with a molecular mass of ~57 kDa, the expected molecular mass of $DT_{cl}GMCSF$ as deduced from its nucleic acid sequence (Fig. 2). The integrity of expression of both the diphtheria toxin and GMCSF moieties of $DT_{cl}GMCSF$ was confirmed in immunoblot analysis employing antisera to diphtheria toxin or GMCSF (Figure 2). In vitro analysis of the catalytic activity of $DT_{cl}GMCSF$ demonstrated the characteristic lethal enzymatic activity of diphtheria toxin, the ability to catalyze the ADP-ribosylation of translation factor EF-2 (data not shown).

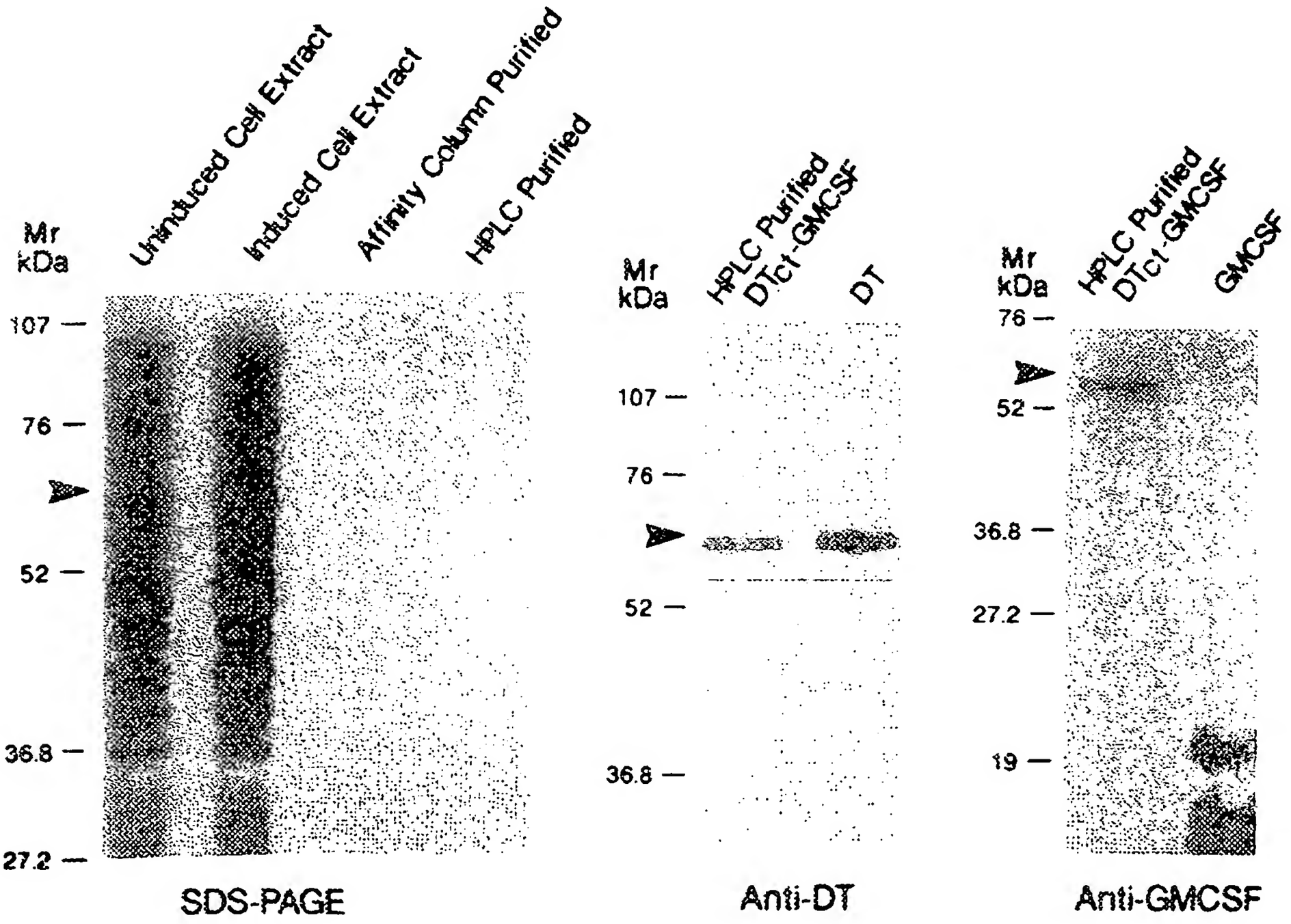


FIGURE 2 Purification of recombinant $DT_{cl}GMCSF$. The expression and sequential anti-DT immunoaffinity and mono-Q HPLC purification of $DT_{cl}GMCSF$ from IPTG-induced 1 liter cultures of E. coli were analyzed by SDS-PAGE, and anti-DT and anti-GMCSF immunoblots, both detecting the 58 kDa $DT_{cl}GMCSF$ protein. The migration of prestained molecular weight size standards is indicated, and for immunoblot analysis, native DT and human GMCSF were used as controls.

GMCSF-R—Specific Cytotoxic Activity of DT_{ct} GMCSF Against Myeloid Leukemia Cells Multiple studies have revealed that GMCSF receptors are expressed on a wide range of myeloid leukemias, including leukemia cell lines such as HL60.[33-37,44] We found that HL-60 cells expressed 77±12 high affinity GMCSF receptors per cell with a K_d of 34.8±3.8 pM (B. Warman and J. Perentesis, unpublished observations). In addition, TF-1 and other leukemia cell lines are dependent upon GMCSF for growth.[32] To complement these previous studies, in this work a variety of leukemia cell lines were examined for their expression of the human GMCSF receptor alpha chain by reverse transcriptase PCR methodology (Fig. 3). These leukemia cell lines were then examined for susceptibility to DT_{ct}GMCSF—mediated cytotoxicity. The cytotoxicity of the fusion toxin was examined using a panel of myeloid leukemia cell lines by employing protein synthesis inhibition assays, a tetrazolium bromide (MTT) assay of cell viability, and clonogenic assays. As shown in Figure 4A, DT_{ct}GMCSF effectively inhibited protein synthesis in the GMCSF-dependent human myeloid leukemia cell line TF-1 with an IC_{50} of approximately 1 ng/ml (=17 pM). DT_{ct}GMCSF was cytotoxic to TF-1 cells in an MTT cell viability assay with a similar IC_{50} value (Fig. 4A). In addition, DT_{ct}GMCSF was selectively cytotoxic to the high-affinity GMCSF-R—bearing human myeloid leukemia cell lines HL-60 and THP-1, as well as the mixed lineage leukemia cell line MV4-11 with IC_{50} values ranging from 1 ng/ml (=17 pM) (HL-60) to 40 ng/ml (=680 pM) (THP-1 and MV4-11) (Fig. 4A). In contrast to DT_{ct}GMCSF, a control fusion toxin, DT_{ct}-interleukin 3 (DT_{ct}IL3), did not kill GMCSF-R—positive HL-60 cells, which do not display the appropriate receptor for IL-3 (Fig. 4B), indicating that the DT_{ct} domain did not possess nonspecific toxicity. Human erythroleukemia cell line K562 and murine leukemia cell line NFS60, neither of which express the high-affinity human GMCSF-R, were not killed by DT_{ct}GMCSF even at 1000 ng/mL concentration (= 17 nM), supporting the notion that the cytotoxicity of DT_{ct}GMCSF was mediated through specific binding to the high-affinity GMCSF-R. Further, the addition of excess native human GMCSF abolished the cytotoxicity of DT_{ct}GMCSF to myeloid leukemia cells, presumably by competition for binding to the GMCSF-R (Fig. 4B). Control studies indicated that GMCSF alone did not stimulate the proliferation or differentiation of HL-60 cells in the DT_{ct}GMCSF treatment groups (data not shown). Taken together, these results provide evidence that recombinant DT_{ct}GMCSF is selectively cytotoxic to GMCSF-R—bearing human leukemia cells and that cytotoxicity depends on high-affinity GMCSF-R binding and internalization.

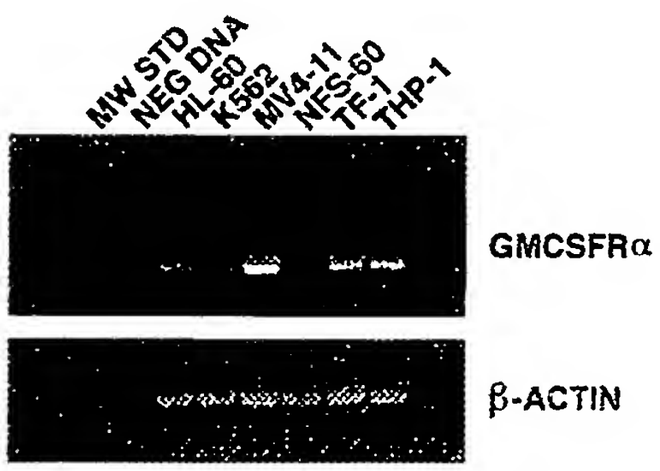


FIGURE 3 Reverse transcriptase PCR analysis of human GMCSF receptor alpha chain expression in mammalian leukemia cell lines using gene-specific primers for receptor mRNA is shown in the *top panel*. Positive controls using human beta-actin primer sets are shown in the *lower panel*. DNA molecular weight size standard and negative control PCR amplification lacking template DNA ("NEG DNA") lanes are indicated.

Cytotoxicity of DT_{ct}GMCSF to Clonogenic Myeloid Leukemia Cells We used a highly sensitive in vitro limiting dilution clonogenic assay system to determine the antileukemic efficacy of DT_{ct}GMCSF against the clonogenic fraction of HL-60 myeloid leukemia cell line. DT_{ct}GMCSF killed clonogenic HL-60 cells in a dose-dependent fashion with >99.99% destruction at 100 ng/mL or 1000 ng/mL (Fig. 5A). Thus, the clonogenic subpopulation of HL-60 myeloid leukemia cells was not spared from the cytotoxic effects of DT_{ct}GMCSF.

Toxicity of DT_{ct}GMCSF to Normal Bone Marrow Progenitors We sought to examine the effects of DT_{ct}GMCSF on in vitro hematopoietic colony formation by normal bone marrow progenitor cells. We postulated that DT_{ct}GMCSF would not inhibit the

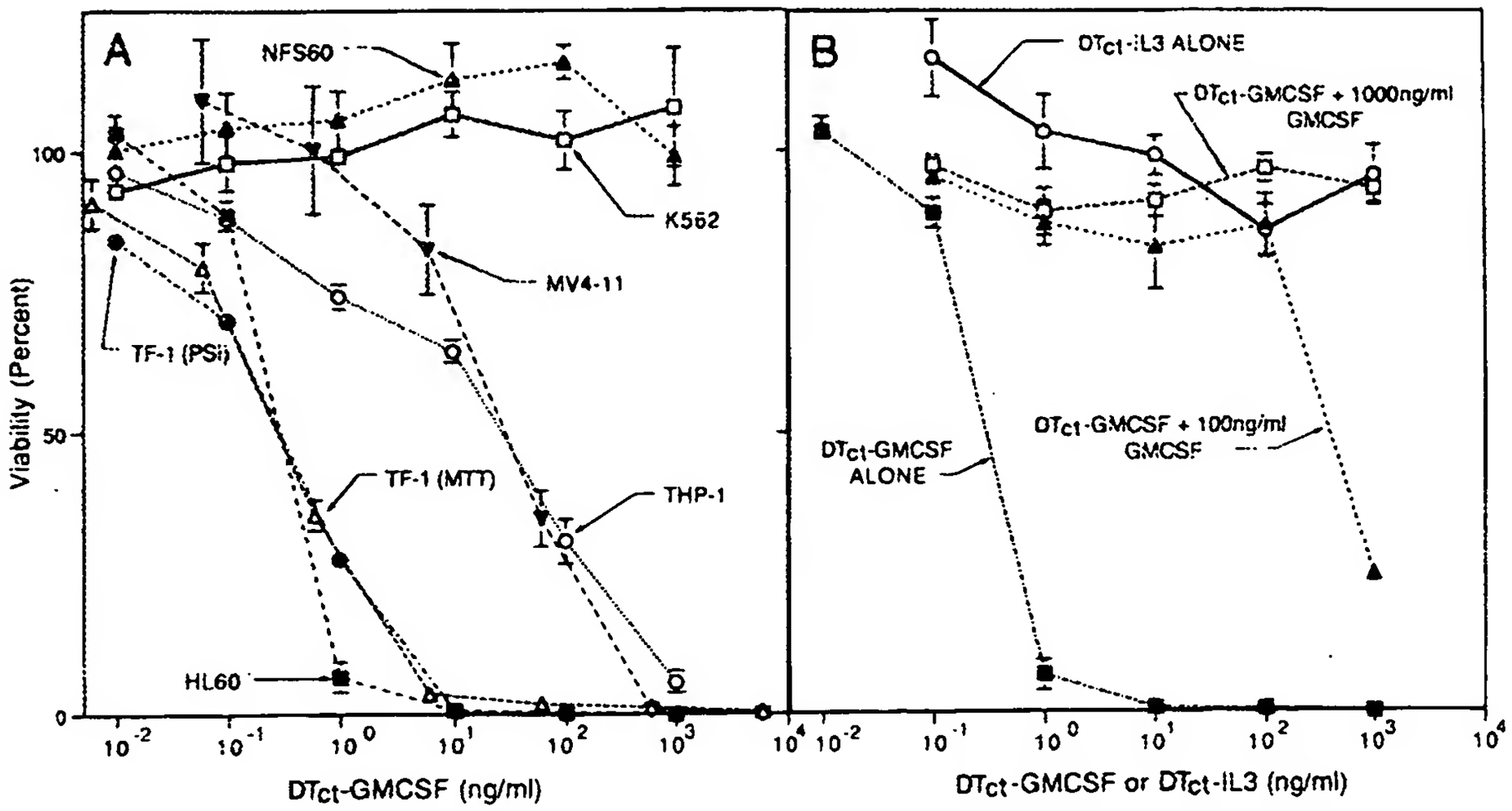


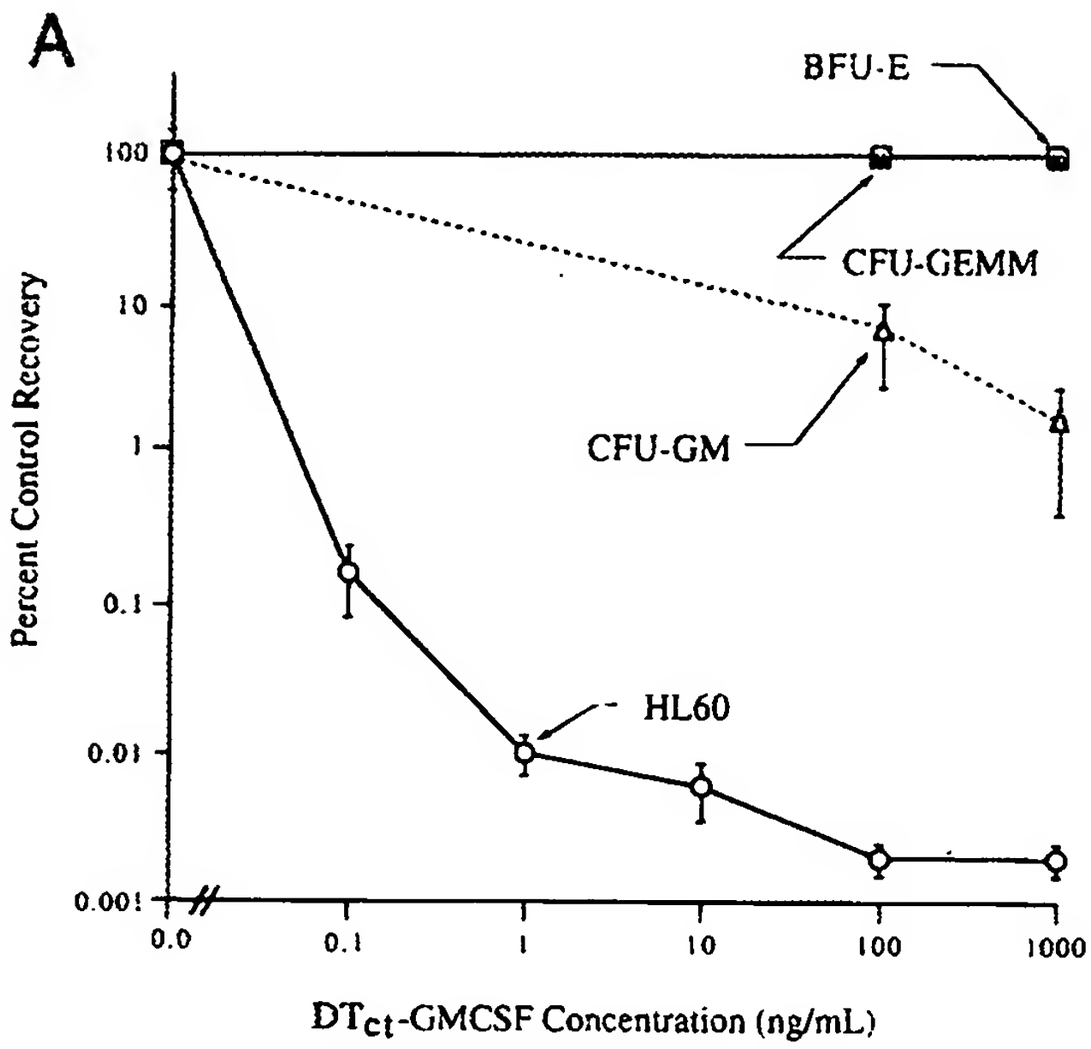
FIGURE 4 Specific $DT_{ct}GMCSF$ protein synthesis inhibition and cytotoxicity to leukemia cells mediated through GMCSF-R binding. (A) Protein synthesis inhibition (PSI) (filled circles) and cytotoxicity (MTT assay) (open triangles) to GMCSF-R—bearing cell human leukemia line TF-1, and cytotoxicity against GMCSF-R—bearing human leukemia cell lines HL-60 (filled squares), THP-1 (open circles), and MV4-11 (inverted open triangles). Controls included GMCSF-R negative human erythroleukemia cell line K562 (open squares) and murine leukemia cell line NFS60 (filled triangles). **(B)** Blocking effects of excess native recombinant GMCSF on the cytotoxicity of $DT_{ct}GMCSF$ against HL-60 cells. $DT_{ct}IL3$ was used as an irrelevant fusion toxin control that does not react with HL-60 cells.

growth of important early pluripotent marrow progenitors or non-myeloid progenitor cells because these progenitor cell populations are not thought to express the high-affinity GMCSF-R. While $DT_{ct}GMCSF$ inhibited granulocyte-macrophage colony formation by committed myeloid progenitor cells (i.e., CFU-GM), it did not affect erythroid burst formation by committed erythroid progenitor cells (i.e., BFU-E), or mixed granulocyte-erythroid-macrophage-megakaryocyte colony formation by pluripotent multilineage progenitor cells (i.e., CFU-GEMM) (Fig. 5A). CFU-GEMM-derived mixed lineage colonies in cultures of $DT_{ct}GMCSF$ treated bone marrow cells were morphologically very similar to mixed lineage colonies in cultures of untreated bone marrow cells and they had a normal size and cellular composition (Fig. 5B). Thus, $DT_{ct}GMCSF$ does not appear to adversely affect the in vitro differentiation capacity of this pluripotent bone marrow progenitor cell population.

DISCUSSION

Diphtheria toxin (DT) kills cells by catalyzing the ADP-ribosylation and consequent inactivation of elongation factor 2 (EF-2), an essential cofactor in protein synthesis, at a unique post-translationally modified histidine residue, diphthamide.[17] EF-2 that has been ADP-ribosylated by DT is unable to interact with ribosomes, leading to an irreversible inhibition of protein synthesis and cell death. The profound toxicity of DT is a result of both its ability to act catalytically, as well as the ubiquitous expression of its receptor, a heparin-binding EGF-like precursor, on human cells.[45] Thus, although DT serves as a prototypic agent to effectively kill cells, its clinical use in unmodified form is precluded because of non-specific toxicity related to the lack of specificity of its receptor-binding domain.

Structural biology studies employing X-ray crystallographic analysis have provided important insight



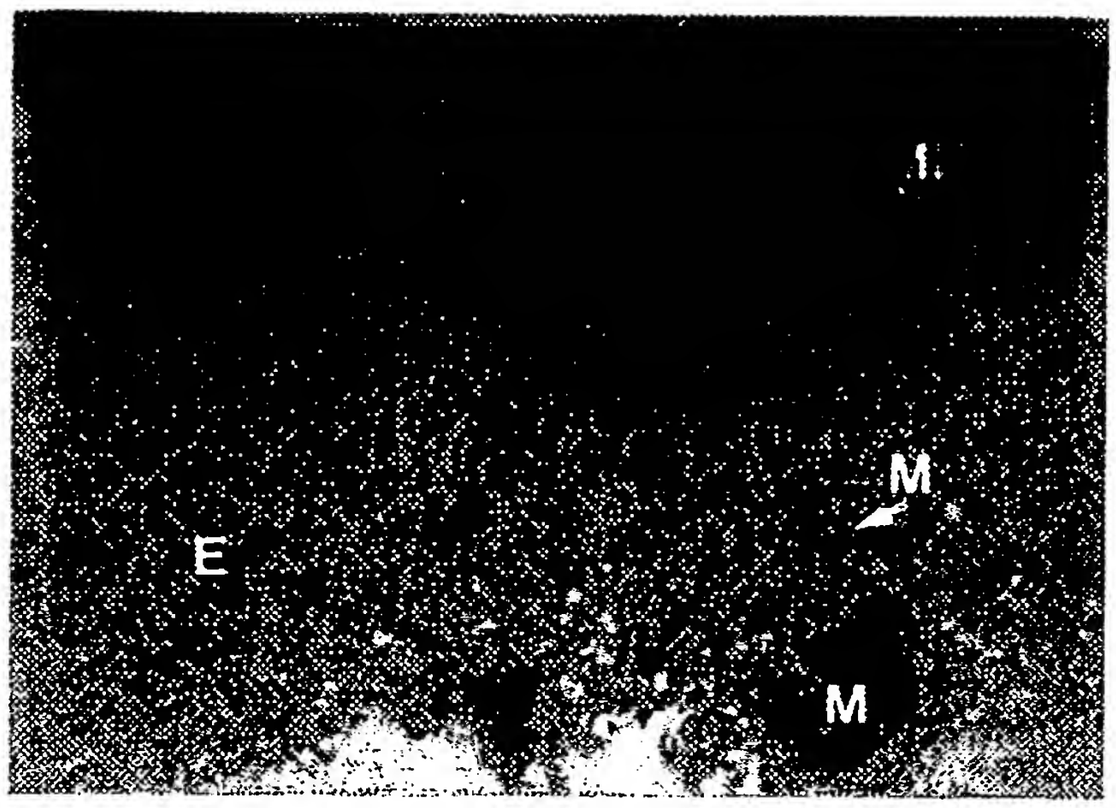


FIGURE 5 (A) Effects of $DT_{\alpha}GMCSF$ on the in vitro clonogenic growth of normal bone marrow progenitor cells vs myeloid leukemia cells. HL-60 cells (open circles) were treated with $DT_{\alpha}GMCSF$ for 4 hours at 37°C, washed twice, and then assayed for clonogenic growth. Bone marrow mononuclear cells from three healthy donors were treated with $DT_{\alpha}GMCSF$ for 24 hours at 37°C, and then assayed for CFU-GM (open triangles) derived granulocyte-macrophage colony formation, BFU-E (open boxes) derived erythroid burst formation, and CFU-GEMM (filled boxes)—derived mixed lineage granulocyte-erythroid-macrophage-megakaryocyte colony formation in methylcellulose cultures. (B) A representative CFU-GEMM-derived mixed lineage granulocyte-erythroid-macrophage-megakaryocyte colony in methylcellulose cultures of $DT_{\alpha}GMCSF$ (100 ng/mL × 24 hours)—treated normal bone marrow mononuclear cells. E, erythroid cells; G, granulocyte; M, macrophage(s); MK, megakaryocyte. (See Color Plate XVI at the back of this issue.)

into the structure-function relationships of DT.[18] Recent refinement of the DT X-ray crystallographic structure has revealed that amino acid residues 380–386, located in a small loop separating the carboxyl terminal receptor binding domain (the "R" domain) from the rest of the toxin (the catalytic "C" and translocation "T" domains), allows the entire 15 kDa binding domain to flexibly rotate as a unit by 180°, with atomic movement of up to 65 Å.[19] Rotation of the binding domain, permits DT dimerization through noncovalent interactions of the binding domain of one DT molecule with the catalytic and translocation domains of a second DT molecule, and vice versa. In separate studies, the elucidation of the crystal structure of GMCSF,[46,47] has demonstrated that it is a member of the four-helix bundle family of cytokines,[48] and defined critical domains that are essential for high-affinity binding to its receptor (GMCSF-R).

We have exploited the identification of the distinct flexible molecular hinge separating the native binding domain ("R" domain) of DT from its catalytic ("C" domain) and translocation domains ("T" domain) to design and produce a novel fusion toxin, DT_{ct}GMCSF, directed against the high-affinity GMCSF-receptor (GMCSF-R) on myeloid leukemia cells. In DT_{ct}GMCSF, the native binding domain of DT is genetically replaced with GMCSF at the site of the flexible molecular hinge. This rational drug design of DT_{ct}GMCSF was intended to preserve essential structure-function relationships identified in crystallographic analyses of both the DT and GMCSF molecules.

A variety of studies have suggested that the high-affinity GMCSF-R is an attractive target for the delivery of specific biologic therapies in the treatment of AML. GMCSF is a hematopoietic growth factor with a pivotal role in the proliferation, differentiation, and function of the myeloid compartment in the human lymphohematopoietic system.[49–51] The heterodimeric high-affinity GMCSF-R belongs to a recently described supergene family, and is composed of an alpha chain specific for GMCSF and a beta chain that can also associate with the interleukin 3 and interleukin 5 receptor alpha chains.[52] AML is a heterogeneous disease and it appears that the vast majority of patients exhibit leukemic cell growth related to the dysregulation of expression of hematopoietic growth factors.[7–10] Further, functional autocrine or paracrine loops specifically involving GMCSF appear to play a central role in the etiology of myeloid leukemia, with frequent activation of these GMCSF-related growth loops caused by other cytokines such as interleukin 1.[10–12] Evidence to support the prominent role of GMCSF in AML leukemogenesis can be seen in studies in which the autonomous proliferation of AML blasts in culture was abrogated in over 80% of patient samples by the presence of either neutralizing GMCSF antibody or an antisense oligonucleotide directed against GMCSF.[13] The GMCSF-R may be expressed on additional cases of myeloid leukemia, and thus effectively function as a pseudo-"leukemia marker", in a manner analogous to the expression of the Tac molecule on T-lineage lymphoid malignancies. Our studies have demonstrated that recombinant DT_{ct}GMCSF is selectively cytotoxic to GMCSF-R bearing human leukemia cells and that cytotoxicity depends on high-affinity GMCSF-R binding and internalization.

The hGMCSF receptor is composed of an alpha chain that is specific for hGMCSF and a beta chain that is shared with IL3 and IL5. The alpha chain alone binds hGMCSF with low affinity, but interaction of hGMCSF with both the alpha and beta chain results in formation of a high-affinity (slowly dissociating) complex followed by internalization of the ligand. Human GMCSF and its receptor play a key role in the proliferation and differentiation of myeloid lineage cells, but the distribution and timing of expression of the high-affinity hGMCSF receptor in various hematopoietic precursors, particularly the pluripotent stem cell and its early progeny, have yet to be fully elucidated. Our studies demonstrated that DT_{ct} GMCSF effected modest inhibition of committed BFU-E and CFU-GEMM, with greater inhibition of CFU-GM marrow precursors, and are in accord with recent observations indicating that the high-affinity GMCSF receptor does not appear to be expressed on the surface of early pluripotent lymphohematopoietic stem cell populations.[53] DT_{ct}GMCSF will be a useful

reagent to directly determine cellular expression of the high-affinity hGMCSF receptor because its cytotoxic effect is dependent only on a functional high-affinity hGMCSF receptor, and we are currently evaluating its effects of on the formation of long term bone marrow culture initiating cells (LT-CIC). Also, DT_{ct}GMCSF will be useful to study the nature and role of the expresson of hGMCSF receptors that have been identified on some non-hematopoietic solid tumors.[54–56]

Studies in progress are directed to definition of the efficacy and toxicity profile of DT_{ct}GMCSF after *in vivo* administration to SCID mice bearing human myeloid leukemias. Furthermore, DT_{ct}GMCSF could also prove useful in the treatment of some non-hematologic forms of cancer because of the potential aberrant expression of high-affinity GMCSF-R on malignant cells from solid tumors, including melanoma, small-cell carcinoma of the lung, breast cancer, and colon cancer.[54–56]

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A Murine Cytokine Fusion Toxin Specifically Targeting the Murine Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) Receptor on Normal Committed Bone Marrow Progenitor Cells and GM-CSF–Dependent Tumor Cells

By Chung-Huang Chan, Bruce R. Blazar, Cindy R. Eide, Robert J. Kreitman, and Daniel A. Vallera

A fusion protein was synthesized consisting of the murine granulocyte-macrophage colony-stimulating factor (mGM-CSF) gene spliced to a truncated form of the diphtheria toxin (DT_{390}) gene coding for a molecule that retained full enzymatic activity, but excluded the native binding domain. The DT_{390}-mGM-CSF hybrid gene was cloned into a vector under the control of an inducible promoter and the protein expressed in *Escherichia coli*. After induction, a protein was purified from inclusion bodies in accord with the deduced molecular weight of DT_{390} mGM-CSF. Cell-free studies of the adenosine diphosphate–ribosylating activity of DT_{390} mGM-CSF showed results that were similar to those of native DT. The DT_{390} mGM-CSF immunotoxin inhibited FDCP2.1d, a murine myelomonocytic tumor line expressing the GM-CSF receptor with an IC_{50} (concentration inhibiting 50% activity) of 5×10^{-11} mol/L. The fusion toxin was specifically cytotoxic and directed by the GM-CSF portion of the molecule because addition of a monoclonal antibody directed against GM-CSF inhibited its ability to kill the cell line. Cell lines that do not express GM-CSF receptor were not inhibited by the fusion toxin. DT_{390} mGM-CSF was also able to specifically inhibit normal committed bone marrow (BM) progenitor cells as measured in clonal colony-forming unit granulocyte-macrophage assays. Together, these findings indicate that DT_{390} mGM-CSF may be useful as a novel tool for purging BM of contaminating leukemia cells or in vivo for eliminating residual leukemia cells. Also, it can be used to determine whether committed and/or noncommitted BM progenitor cells express the GM-CSF receptor.

MURINE granulocyte-macrophage colony-stimulating factor (mGM-CSF) is composed of 124 amino acids with a calculated molecular weight of 14.138 kD.[1] GM-CSF can stimulate proliferation and differentiation of colony-forming unit–granulocyte-macrophage (CFU-GM) progenitor cells as well as enhance the function of mature neutrophils, monocytes, and eosinophils.[2] Exposure of CFU-GM progenitors to GM-CSF causes rapid entry of cells into the cell cycle. The biologic actions of GM-CSF are mediated by binding to a specific high-affinity receptor, consisting of two components designated as α and β subunits.[3,4] These GM-CSF receptors are found on the surface of myeloid precursors, granulocytes, mononuclear phagocytes,[5-7] and also frequently present on the myeloid malignancies.[8-10] In fact, the majority of cases of acute myeloid leukemia express high-affinity receptors for GM-CSF.

GM-CSF can interact with myeloid leukemic cells because GM-CSF, either by itself or in association with other cytokines, can induce proliferation of myeloid clonogenic blasts.[11-13] Several reports suggest that GM-CSF receptors are expressed on human nonhematopoietic tumor cell lines[14-17] and GM-CSF is capable of stimulating the growth of human solid tumor cell lines including those derived from small cell lung carcinoma, melanoma, renal carcinoma, colon carcinoma, gastric carcinoma, and ovarian carcinoma.[14,18-20]

The first step in the proliferative action of GM-CSF on leukemic progenitors is ligand binding to specific membrane receptors.[5,6,21,22] The receptor is composed of two subunits and the binding of these α and β subunits together form a high-affinity (dissociation constant [kd], approximately 40 pmol/L) receptor complex.[5] The binding of GM-CSF to this receptor causes rapid internalization of the ligand-receptor complex.[23] Because of the internalization of GM-CSF, we reasoned that GM-CSF could serve as a ligand for delivering a toxic molecule such as diphtheria toxin (DT) to myeloid leukemic cells.

DT is a well-studied glycoprotein with a molecular weight of 58 kD. DT has potent cell-killing ability and requires internalization.[24] Its mechanism involves adenosine diphosphate (ADP)-ribosylation of elongation factor-2, resulting in inhibition of cellular protein synthesis and death of the cell. Investigators have shown that DT induces DNA degradation and morphologic changes consistent with apoptosis.[25] Delivering a single DT molecule into the cytoplasm is sufficient to kill a cell.[26] Native DT contains three domains: the cell-binding domain, the translocation domain, and the enzymatic cytotoxic domain.[26-28] The cell-binding domain of the DT gene can be replaced by a growth factor gene, resulting in a toxin-growth factor hybrid gene, whose protein product is targeted to a specific growth-factor receptor. Fusion toxins have been reported that specifically target DT to cytokine receptors including interleukin-2 (IL-2), IL-4, IL-6, and G-CSF receptors.[29-33]

We constructed a fusion toxin targeting cells bearing the mGM-CSF receptor (1) to devise a reagent that is potentially useful in destroying the residual myeloid leukemic cells dur-

From the Department of Therapeutic Radiology, Section on Experimental Cancer Immunology, the Department of Laboratory Medicine and Pathology, and the Department of Pediatrics, Division of Bone Marrow Transplantation, University of Minnesota Hospital and Clinics, Minneapolis, MN; and the National Cancer Institute, National Institutes of Health, Bethesda, MD.

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Address reprint requests to Daniel A. Vallera, PhD, Box 367 UMHC, Harvard St at E River Rd, Minneapolis, MN 55455.

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ing bone marrow (BM) purging or that could be used for in vivo therapy of residual disease or (2) to explore the expression of GM-CSF receptor on noncommitted and committed BM progenitor cells. In this report, we describe the construction of the novel DT_{390} mGM-CSF fusion toxin consisting of the murine GM-CSF gene spliced to a truncated form of the DT gene that retains enzymatic activity, but excludes the native binding domain. We investigated the potency and specificity of our construct in its reactivity with murine GM-CSF receptor-expressing cell lines and normal granulocyte-macrophage BM progenitor cells.

MATERIALS AND METHODS

Construction of hybrid gene and plasmid. Oligonucleotides were synthesized using cyanomethyl phosphoramidite chemistry on an Applied Biosystems model 380 A DNA synthesizer and purified by chromatography on Oligonucleotide Purification Cartridges (Applied Biosystems, Foster City, CA) as recommended by the manufacturer. Purified oligonucleotides were resuspended in TE buffer (10 mmol/L TRIS base, 1 mmol/L EDTA, pH 8.0). The sequences of oligonucleotides used in this study are listed in Table 1. The hybrid gene encoding DT_{390} mGM-CSF was constructed by the method of gene splicing by overlap extension (SOE) as described.[34] Briefly, a DT gene fragment was generated in the first polymerase chain reaction (PCR) by using 5.5 ng plasmid containing the cDNA of DT mutant CRM107 as a template with primers a and b (Table 1). Primer a created an *Nco* I restriction site and an ATG initiation codon 5′ to the DT coding sequence. Primer b introduced a coding sequence for a linker [$(Gly_4Ser)_4$] directly after amino acid 389 of the mature DT molecule. A murine GM-CSF gene fragment was generated in the second PCR by using 1.8 ng plasmid containing the cDNA of murine GM-CSF as a template with primers c and d. Primer c created sequence homology with the linker at the 3′ end of the DT fragment generated in the first PCR. This region of homology was placed 5′ to the sequence encoding amino acid 26 of the GM-CSF molecule. Primer d introduced an *Xho* I restriction site at the end of the GM-CSF molecule. The two fragments generated in the PCRs described above were then purified and used as templates in a SOE reaction using primers a and d. This SOE formed the full-length DT_{390}–mGM-CSF hybrid gene. The DT_{390}–mGM-CSF hybrid gene was digested with restriction enzymes *Nco* I and *Xho* I (GIBCO BRL, Gaithersburg, MD) and ligated into the *Nco* I and *Xho* I cloning sites in the pET21d plasmid (Novagen, Madison, WI). The assembly of plasmid pDTGM-CSF is shown in Fig 1.

Expression and localization of fusion proteins. Plasmid, pDT GM-CSF, was transformed into the *E coli* strain BL21(DE3) (Novagen, Madison, WI) and protein expression was evaluated. Briefly, recombinant bacteria were grown in 500 mL Luria broth, supplemented with 100 μg/mL carbencillin (Sigma Chemical Co, St Louis, MO), in a 2-L flask at 37°C. When the absorbance (A_{600}) of culture reached 0.6, expression of the hybrid gene was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) (GIBCO BRL). Three hours after induction, the bacteria were obtained by centrifugation at 5,000*g* for 10 minutes. To determine the localization of expressed protein, an aliquot of bacterial pellet was resuspended in 30 mmol/L TRIS, pH7.5, 20% sucrose, 1 mmol/L EDTA and osmotically shocked by placing in ice-cold 5 mmol/L $MgSO_4$. The periplasmic fraction (supernatant) was obtained by centrifugation at 8,000*g* for 10 minutes. Another aliquot of bacterial pellet was resuspended in sonication buffer (50 mmol/L sodium phosphate, pH 7.8, 300 mmol/L NaCl). After incubation at −20°C for 16 hours, the resuspended sample was sonicated for 5 minutes. The spheroplast fraction (pellet) and cytosolic fraction (supernatant) were collected separately by centrifugation at 10,000*g* for 20 minutes.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. Crude and purified fusion proteins were analyzed on SDS-PAGE. SDS-PAGE was performed using 4% to 20% gradient gels (Bio-Rad, Richmond, CA) and a Mini-Protein II gel apparatus (Bio-Rad). Proteins were stained with Coomassie brilliant blue. For immunoblotting, electrophoresed proteins were transferred to nitrocellulose membranes. Membranes were blocked with 3% gelatin-containing TBS (20 mmol/L TRIS, 500 mmoL NaCl, pH 7.5) and washed with TTBS (TBS, 0.05% Tween-20, pH 7.5). Horse anti-DT antisera (Connaught Lab, Switwater, PA) and anti–GM-CSF (a rat IgG monoclonal antibody from clone 22E9.11 generously supplied by Dr John Abrams (DNAX, Palo Alto, CA) were used as a source of primary antibodies. The blots were processed using horseradish peroxidase–conjugated protein-G (protein G-HRP) and developed using HRP color reagents (Bio-Rad, Richmond, CA).

Isolation of inclusion bodies and purification of the fusion proteins. To isolate expressed protein from inclusion bodies, a bacterial pellet was resuspended in TE buffer (50 mmol/L TRIS, pH 8.0, 20 mmol/L EDTA, 100 mmol/L NaCl) and treated with 5 mg/mL lysozyme for 30 minutes. The pellet was then incubated in Triton X-100 buffer (11% vol/vol Triton X-100, 89% vol/vol TE) for 30 minutes at room temperature after briefly homogenizing with a tissuemizer (Thomas Scientifics, Staufen, Germany). Inclusion bodies were collected by centrifugation at 24,000*g* for 50 minutes. Solubilization of the inclusion body pellet was achieved in the presence of strong denaturants and reducing agents in a buffer consisting of 7 mol/L guanidine, 0.1 mol/L TRIS, pH 8.0, 2 mmol/L EDTA, and 65 mmol/L dithioerythritol. The solution was incubated at room temperature for 16 hours. To remove insoluble material, the solution was centrifuged at 40,000*g* for 10 minutes. Protein concentrations were determined according to Bradford method.[35] To ensure proper

Table 1. Sequence of Oligonucleotides Used in This Study

Primer	Characteristics	Sequence
a	The sense primer introduced an *Nco* I site with an initiation codon ATG and the initial 7 codons of DT.	5′AGATATA**CCATGG**GCGCTGATGATGTTGTTGAT3′
b	The antisense primer introduced the codons 384 to 389 of DT and codons of a linker [(Gly)4Ser]4.	5′*CGACCCACCACCGCCCGAGCCACCGCCAC CGCTTCCACCGCCTCCAGATCCGCCGCCACC* AAATGGTTGCGTTTTATG3′
c	The sense primer introduced the codons 26 to 31 of GM-CSF and codons of part of the linker.	5′*TCGGGCGGTGGTGGGTCG* GTCACCCGGCCTTGGAAG3′
d	The antisense primer introduced an *Xho* I site and the last 7 codons of GM-CSF.	5′CGTGCG**CTCGAG** TTTTTGGCTTGGTTTTTTGCA3′

The sequence of the restriction site is in bold text. The sequence of the [$(Gly_4Ser)_4$] linker is in italic text.

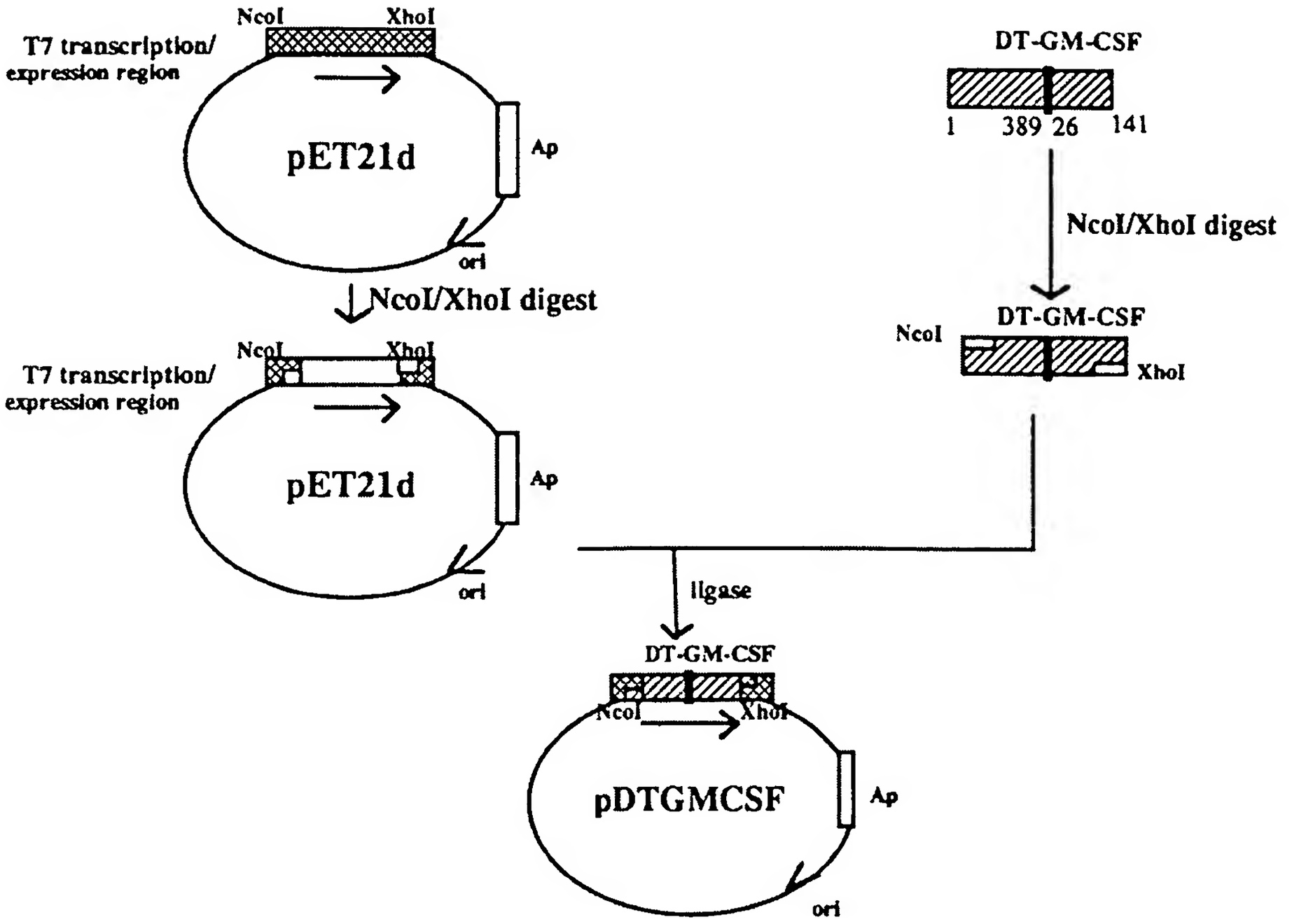

Fig 1. Assembly of the DT_{390}–mGM-CSF gene in the pET21d vector. The hybrid gene encoding DT_{390} mGM-CSF was constructed by the method of gene splicing by SOE. The DT_{390} mGM-CSF hybrid gene was digested with restriction enzymes *Nco* I and *Xho* I and ligated into the *Nco* I and *Xho* I cloning sites of a pET21d plasmid under the control of a T7 promoter.

tertiary folding, renaturation was initiated by a rapid 100-fold dilution of the denatured and reduced protein into refolding buffer consisting of 0.1 mol/L TRIS, pH 8.0, 0.5 mol/L L-arginine, 0.9 mmol/L oxidized glutathione, and 2 mmol/L EDTA. The samples were incubated at 10°C for 48 hours. The refolded protein was diafiltrated and ultrafiltrated against 20 mmol/L TRIS, pH 7.8, using a spiral membrane ultrafiltration cartridge on Amicon's CH2 system (Amicon, Beverly, MA). Samples were loaded on a Q-Sepharose (Sigma) column and eluted with 0.3 mol/L NaCl in 20 mmol/L TRIS, pH 7.8. The protein was diluted fivefold and subsequently applied to another Q-Sepharose column and eluted with a linear salt gradient form 0 to 0.4 mol/L NaCl in 20 mmol/L TRIS, pH 7.8. The main peak from the second Q-Sepharose column was purified by size-exclusion chromatography on a TSK 250 column (Toso Hass, Philadelphia, PA).

ADP ribosylation assay. The toxin was nicked by treating 15 μg of DT_{390} mGM-CSF with 0.04 μg of trypsin for 15 minutes at 37°C. The reaction was stopped with soybean inhibitor. Duplicate samples of nicked DT and DT_{390} GM-CSF were examined for their ADP ribosyl transferase activity as previously described.[36] Briefly, ADP-ribosylation was performed in 80-μL reaction mixtures containing 50 μL of 0.01 mol/L TRIS-HCl buffer with 1.0 mmol/L dithiothreitol, pH 8.0, 10 μL of rabbit reticulocyte lysate (containing the elongation factor 2, (EF-2), and 10 μL of toxin sample. The reaction was initiated by the addition of 10 μL of 0.57 mmol/L [^{32}P] nicotinamide adenine dinucleotide (ICN Biomedicals, Irvine, CA). Reaction mixtures were incubated at room temperature for 1 hour and the reaction was stopped by the addition of 1 mL 10% trichloroacetic acid (TCA). The precipitate was collected by centrifugation and washed with 1 mL 10% TCA. The radioactivity was counted by standard scintillation techniques.

Cytotoxicity assay. To characterize the cytotoxic activity of DT_{390} mGM-CSF, we used the murine myelomonocytic cell line FDCP2.1d (provided by Immunex Inc, Seattle, WA), which is dependent on mGM-CSF for proliferation. Cultured FDCP2.1d cells were maintained in complete culture media consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 1% sodium pyruvate, 1.5% L-glutamine, 2% penicilliin/streptomycin, 2% HEPES, 0.8% L-arginine, 2% folic acid and L-asparigine, and exogenous mGM-CSF at a final concentration of 1 ng/mL. We assayed the cytotoxic activity by measuring the ability of DT_{390} mGM-CSF to inhibit the proliferation of FDCP2.1d cells. Cells were initially washed twice with plain DMEM to remove the exogenous cytokine followed by a 1-hour incubation at 37°C. A third wash was performed and the cells were resuspended. Cells were seeded at a concentration of 9×10^4 cells per tube in complete culture media and treated with the following toxins: DT_{390} mGM-CSF, DT_{390} hIL-2, DT_{390} mIL-4, native DT, and ricin at concentrations ranging from 1×10^{-11} mol/L to 1×10^{-8} mol/L. The cells were treated for 4 hours at 37°C in a 5% CO_2 atmosphere. After incubation, the tubes were centrifuged for 10 minutes at 300*g* and the supernant decanted. The cells were washed with complete media three times and resuspended with 600 μL media after the third wash. Cells were seeded at 3×10^4 cells/well in 96-well flat-bottomed

plates in a volume of 200 μL. One microcurie [H^3]-thymidine and exogenous mGM-CSF at final concentration of 1 ng/mL were added into each well. After a 24-hour incubation, the cells were procured on glass fiber filters. Filters were washed, dried, and counted according to standard methods suggested by manufacturer. Cells cultured with media alone served as the control. All assays were performed in triplicate. Two additional control murine cell lines EL4, a T-cell leukemia/lymphoma and the myeloid leukemia C1498 (American Type Culture Collection, Rockville, MD) were used that did not respond to mGM-CSF.

Colony-forming assay. DT_{390} mGM-CSF, DT_{390} mIL-2, and native DT were examined for their effects on BM cells in a colony-forming assay, which was performed by short-term culture of toxin-treated murine BM cells in complete methylcellulose media (30% fetal calf serum, 1% pokeweed mitogen–stimulated murine spleen cell conditioned medium, 1% bovine serum albumin (BSA), 0.9% methylcellulose, 10^{-4} mol/L 2-mercaptoethanol, and 3 U/mL erythropoietin) (StemCell Technologies, Vancouver BC, Canada). Briefly, BM cells were collected into RPMI 1640 media by flushing the shafts of femora and tibia of C57BL/6 mice. Cells were resuspended at 5×10^4 cells/mL in complete methylcellulose media with toxin at a final concentration of either 10^{-8} or 10^{-9} mol/L and were plated and incubated in culture dishes for 14 days under fully humidified conditions in an atmosphere of 5% CO_2 at 37°C. Under an inverted microscope, colonies of greater than 50 cells were scored as CFU-GM according to their morphology. All assays were performed in duplicate.

RESULTS

Genetic construction of DT_{390} mGM-CSF. The DNA fragments encoding the structural gene for DT_{390} and mGM-CSF were obtained by separate PCRs with the sizes of 1,239 and 380 bp, respectively. After the third PCR, the resulting SOE product, DT_{390} mGM-CSF hybrid gene, was generated with 1,601-bp size. The DT_{390} mGM-CSF hybrid gene encodes an *Nco* I restriction site, an ATG initiation codon, the first 389 amino acids of DT, a 20–amino acid interchain spacer, the mature murine GM-CSF polypeptide, and an *Xho* I restriction site. After digestion, the DT_{390}–mGM-CSF hybrid gene was cloned into the pET21d plasmid under the control of the IPTG-inducible T7 promoter to create pDT GM-CSF. Restriction endonuclease digestion and DNA sequencing analysis were used to verify that DT_{390} mGM-CSF hybrid gene sequence had been cloned in frame (data not shown).

The plasmid was sequenced by the University of Minnesota Microchemical Facility (University of Minnesota, Minneapolis). The junctional region was found to encode for amino acids 333 to 389 of DT with one substitution of alanine at postion 356 by serine. The linker was found to encode for the following amino acids, $[(Gly)_4Ser]_2GlyAla(Gly)_2Ser(Gly)_4SerPhe$ joined by a sequence encoding amino acids 27 through 175 of mGM-CSF.

Expression and purification of DT_{390}–mGM-CSF fusion protein. Expression of the fusion protein in *E coli* was induced with IPTG at 37°C. Coomassie blue–stained SDS-polyacrylamide gel of whole bacterial lysate post-IPTG induction showed a protein migrating at 58 kD, which corresponds to the expected size for DT_{390}–mGM-CSF protein. The localization study of the expressed fusion protein showed that DT_{390} mGM-CSF was retained in the inclusion bodies (Fig 2). To extract the DT_{390}–mGM-CSF protein, the inclusion bodies were isolated, denatured, and refolded as described in Materials and Methods. After the renaturation procedure, the crude DT_{390} mGM-CSF was purified by sequential chromatography. The elution from the anion-exchange Q-sepharose column showed an enrichment of a protein with an electrophoretic mobility corresponding to an apparent molecular mass of 58 kD (Fig 2, lane 6). To further purify this fusion protein, pooled peak fractions from the anion-exchange Q-sepharose column were subjected to high-performance liquid chromatography (HPLC) using a TSK-250 sizing column (Fig 2, lane 7). The final product was 80% pure. To prove that the protein was indeed a fusion of of this DT_{390} spliced to mGM-CSF, further analysis was performed by immunoblotting. Anti-DT antiserum was able to recognize the DT_{390} mGM-CSF, both in the crude bacterial lysate and after renaturation (Fig 3). It is of interest to note that anti–mGM-CSF antiserum was able to recognize the DT_{390} mGM-CSF only after proper renaturation.

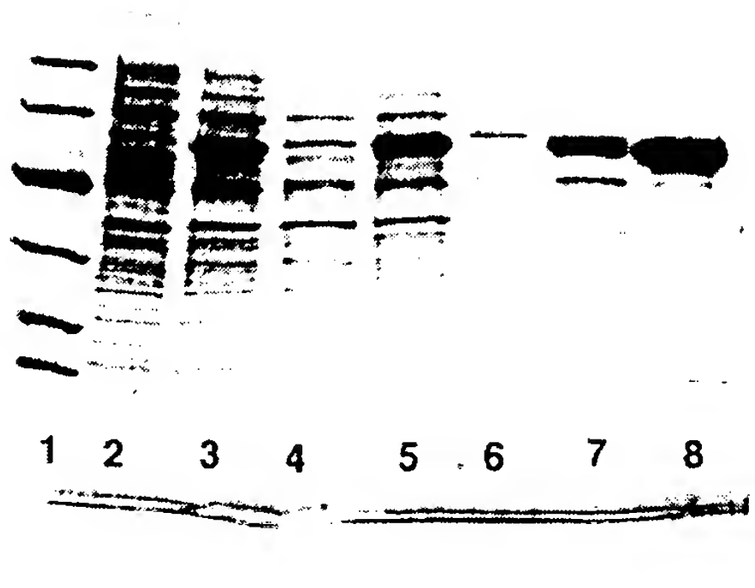

Fig 2. SDS-PAGE of the purified DT_{390}–mGM-CSF protein stained with Coomassie Blue. Lane 1, molecular weight standard; lane 2, uninduced total bacteria lysate; lane 3, induced total bacteria lysate; lane 4, fraction of soluble protein in cytoplasm; lane 5, fraction of insoluble protein in inclusion body; lane 6, soluble protein in periplasmic space; lane 7, major elution of anion-exchange column; and lane 8, major peak of HPLC sizing column. The molecular standards migrated (beginning with the largest protein) at 97, 66, 45, 31, 22, and 14 kD.

Enzymatic activity and in vitro cytotoxicity. Protein synthesis inhibition by DT is caused by fragment A–catalyzed ADP-ribosylation of cytoplasmic EF-2. To determine whether the DT_{390}–mGM-CSF protein also displays such enzymatic activity, a cell-free assay system was used, in which rabbit reticulocyte lysate, a source of EF-2, was exposed to either native DT or DT_{390} mGM-CSF in the presence of [^{32}P]-nicotinamide adenine dinucleotide. Incubation with either toxin showed a similar dose-dependent increase in [^{32}P] incorporation into the TCA-precipitable fraction (Fig 4).

Fig 3. Western blot analysis of DT_{390}–mGM-CSF protein. (A) Nitrocellulose probed with polyclonal anti-DT serum. Lane 1, renatured protein after anion-exchange column; lane 2, induced total bacteria lysate; lane 3, uninduced total bacteria lysate; and lane 4, molecular weight standards. (B) Nitrocellulose probed with anti–mGM-CSF antibody. Lane 1, renatured protein after anion-exchange column; lane 2, induced total bacteria lysate; lane 3, uninduced total bacteria lysate; and lane 4, molecular weight standards.

This result confirmed that DT_{390} mGM-CSF possesses ADP-ribosyl transferase activity.

To characterize the cytotoxic activity of DT_{390} mGM-CSF, a bioassay was devised using the mGM-CSF–dependent myelomonocytic leukemia cell line FDCP2.1d. The cytotoxicity was evaluated by measuring the inhibition of cellular proliferation. The ability of various concentrations of DT_{390} mGM-CSF to inhibit the proliferation on FDCP2.1d cells was examined. FDCP2.1d cells were inhibited by DT_{390} mGM-CSF in a dose-dependent manner with an IC_{50} of 5×10^{-11} mol/L or 3 ng/mL (Fig 5). To determine if the cytotoxic activity of DT_{390} mGM-CSF on FDCP2.1d cells was mediated by the binding of the mGM-CSF moiety, several other toxins including DT_{390} hIL-2, DT_{390} mIL-4, and native DT were

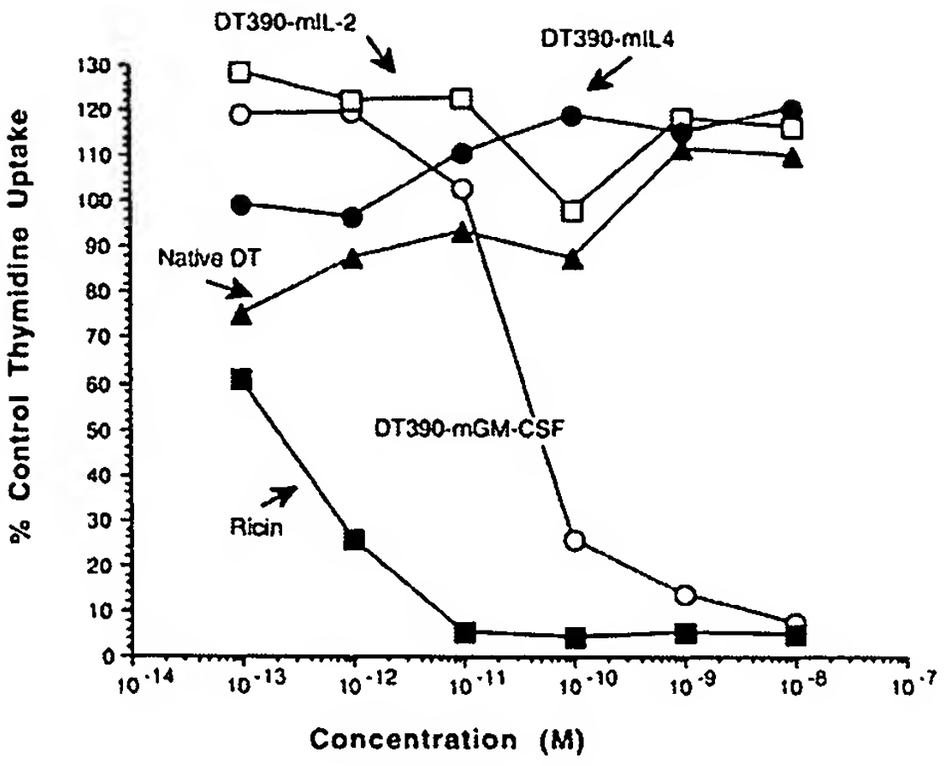

Fig 5. Cytotoxic activity of fusion toxins on FDCP2.1d cells. Fusion toxins were added at various concentrations to FDCP2.1d cells for 4 hours. After washing out toxin, the percent incorporation of [H^3] thymidine relative to untreated controls was determined.

also assayed for their activity against FDCP2.1d. In contrast with DT_{390} mGM-CSF, FDCP2.1d cells were resistant to DT_{390} hIL-2, DT_{390} mIL-4, and native DT up to a concentration of 1×10^{-8} mol/L. Furthermore, anti–mGM-CSF antibodies blocked the cytotoxic effect of DT_{390} mGM-CSF in a dose-dependent manner . Without addition of anti–mGM-CSF antibodies, DT390 mGM-CSF at concentration of 1×10^{-9} mol/L produced a 84% inhibition of cellular proliferation of FDCP2.1d. The addition of 1 nmol/L anti–mGM-CSF antibodies partially neutralized this cytotoxic effect. The addition of 10 nmol/L or 100 nmol/L anti–mGM-CSF antibodies completely neutralized this cytotoxic effect (Fig 6). The addition of 10 nmol/L GM-CSF to 1 nmol/L DT_{390}

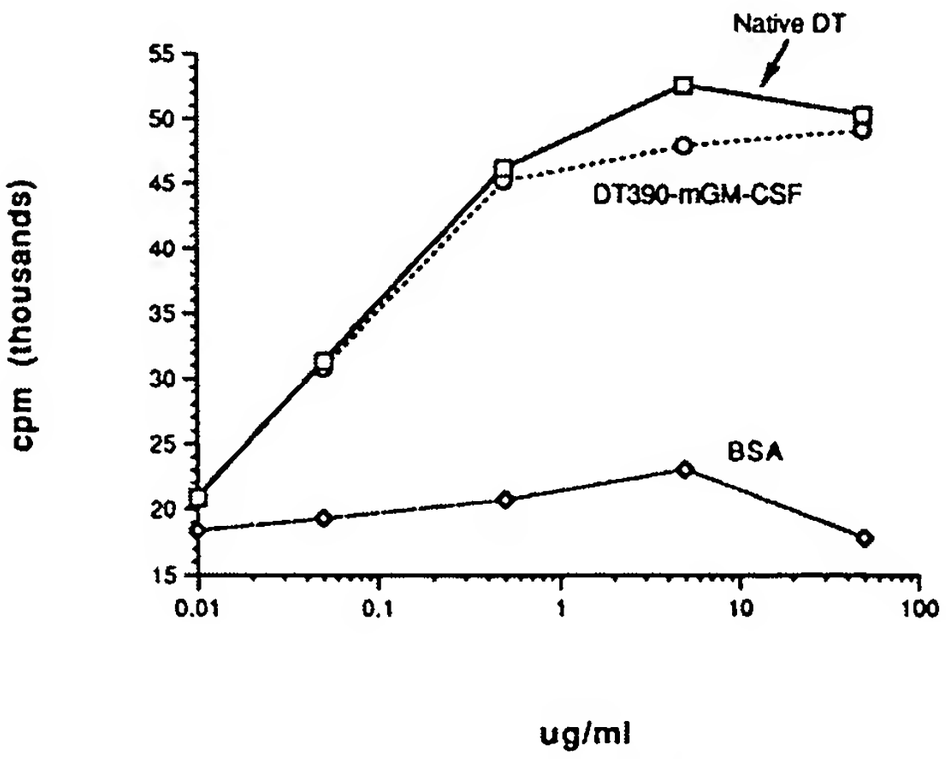

Fig 4. ADP ribosyl transferase activity of DT_{390} mGM-CSF. Trypsin-nicked DT_{390} mGM-CSF, trypsin-nicked native diphtheria toxin, or BSA was studied in cell-free assay. Protein was added at various concentration to the reaction system. The activity was measured as the count of bound ^{32}P-ADP-ribose to rabbit reticulocyte lysate (EF-2).

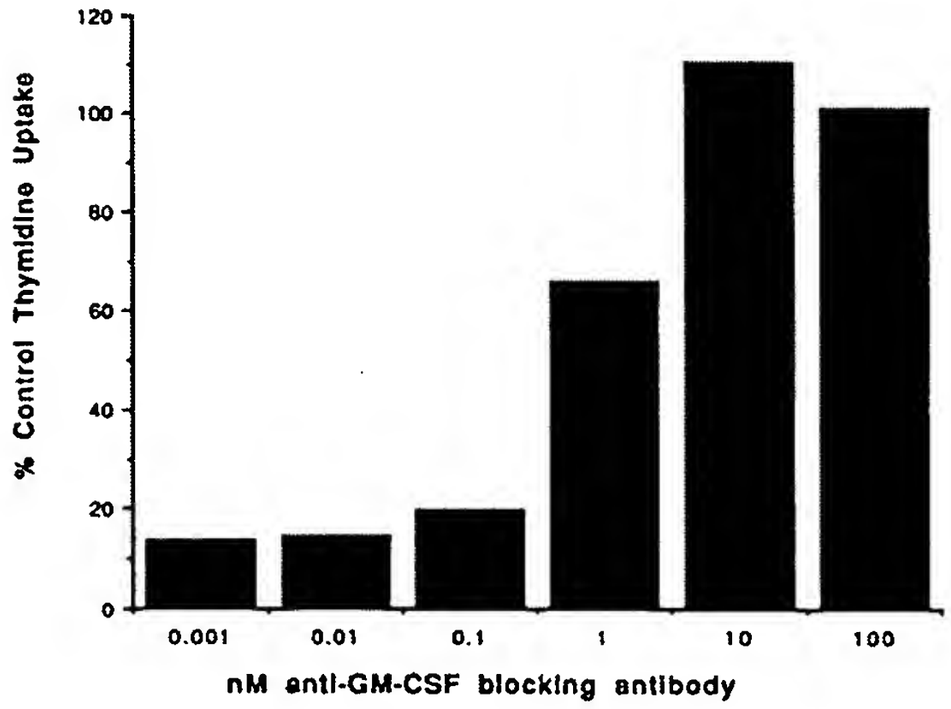

Fig 6. Neutralization of DT_{390} mGM-CSF by anti–mGM-CSF antibody. DT_{390} mGM-CSF (10^{-9} mol/L) was incubated with various concentrations of anti–mGM-CSF or with no antibody and added to FDCP2.1d cells. Results are expressed as the percent of untreated control cells.

mGM-CSF inhibited the response 25%, but the addition of IL-3 did not inhibit. The effect of DT_{390} mGM-CSF on two GM-CSF nonresponsive cell lines was performed to further examine the specificity of the cytotoxic effect. DT_{390} mGM-CSF did not inhibit the T-cell line EL4 or leukemia cell line C1498 (Table 2). Together these data indicate that DT_{390} mGM-CSF is specifically cytotoxic to cells via the GM-CSF ligand-receptor complex.

The effect of DT_{390} mGM-CSF on myeloid progenitor stem cells. It has been well documented that GM-CSF plays a role in the development of the myeloid lineage in hematopoiesis. To test the effect of DT_{390} mGM-CSF on committed myeloid progenitor cells, a colony-forming assay was performed by incubating murine BM cells with toxins including DT_{390} mGM-CSF, DT_{390} hIL-2, and native DT. The DT_{390} mGM-CSF inhibited the formation of CFU-GM up to 90% at the toxin concentrations of 1 nmol/L and 10 nmol/L. In contrast, DT_{390} mIL-2 and native DT had little inhibitory effect on CFU-GM (Fig 7). From these data, we conclude that DT_{390} mGM-CSF has activity against committed myeloid progenitor cells in vitro.

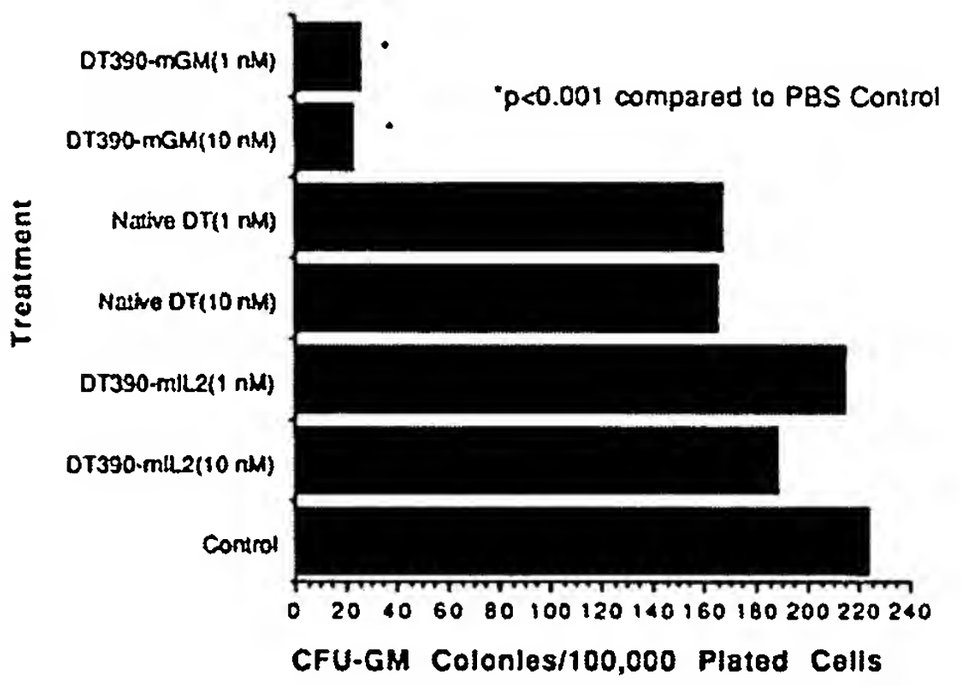

Fig 7. Effect of DT_{390} mGM-CSF on the colony formation of CFU-GM myeloid progenitor cells. Mouse BM cells were incubated with fusion toxin and BM was cultured. Colony number was determined relative to 1×10^5 mononuclear BM cells plated on the semisolid methylcellulose media.

DISCUSSION

The unique contribution of this work is the construction and description of a fusion toxin, DT_{390} mGM-CSF by genetically splicing the DNA segment encoding the ADP-ribosyl transferase enzymatic and hydrophobic translocation enhancing region of DT, but not the native binding site to the DNA segment encoding the amino acids of the mature mGM-CSF molecule. Our data show that the selective binding of this chimeric protein to GM-CSF receptor expressing myelomonocytic leukemia cell lines results in the delivery of a potent and fatal signal that precipitously decreases the proliferation of these cells.

The potency of DT_{390} mGM-CSF was high because we measured an IC_{50} of about 5×10^{-11} mol/L against the cell line FDCP2.1d. Recently, Lappi et al has chemically conjugated human GM-CSF to the ribosome-inactivating protein saporin (SAP).[37] The hGM-CSF-SAP showed an IC_{50} about 3 to 4×10^{-12} mol/L on cell lines transfected with both subunits of GM-CSF receptors. Although these studies involved different toxin moieties, different species of GM-CSF, and different receptor numbers on different cell lines, DT_{390} mGM-CSF was comparable in cytotoxicity to this and other diphtheria-based fusion toxins. For example, DAB_{389} IL-2 and DAB_{486} IL-2 usually show potency with IC_{50} ranging from 2×10^{-11} to 1×10^{-10} mol/L.[38,39] DT_{390} mGM-CSF was specific because it was cytotoxic to mGM-CSF dependent cell line FDCP2.1d, but not the mGM-CSF nonresponsive cell lines C1498 and EL4. DT_{390} mGM-CSF activity was inhibited by an excess of anti–mGM-CSF antibody and, thus, was directed by the GM-CSF portion of the molecule. The IC_{50} of the fusion protein (50 pmol/L) corresponded to the reported dissociation constant for high-affinity GM-CSF binding sites (20 to 60 pmol/L).[30]

Table 2. Sensitivity of Various Cell Lines to DT_{390}-mGM-CSF

Cell Line	Origin	mGM-CSF Response	IC_{50} (mol/L)
FDCP2.1d	Mouse myelomonocytic leukemia	mGM-CSF dependent	5×10^{-11}
C1498	Mouse lymphoma	Negative	$1 \times >10^{-8}$
EL4	Mouse T-cell lymphoma	Negative	$1 \times >10^{-8}$

Cell lines were incubated with DT_{390}-mGM-CSF for 4 hours, washed, pulsed with tritiated thymidine, and then incubated for 24 hours. Cells were harvested onto filters and then counted. Data were plotted as percent control inhibition versus increasing concentration of fusion toxin. IC_{50} dosage (the dose at which 50% of the total response was inhibited) was determined from the curves.

The study was initiated because alternative therapies are still a priority for the treatment of acute nonlymphocytic leukemias, which are still a serious clinical problem. GM-CSF was selected as a ligand because GM-CSF receptor is expressed on these myeloid leukemias.[5-7] It has been postulated that GM-CSF is involved in malignant transformation and metastases because of its expression on neoplastic cells.[12,22,41] Binding GM-CSF causes internalization of GM-CSF receptor in a variety of murine cells at 37°C by receptor-mediated endocytosis.[11,40] Thus, we expected that the GM-CSF component of the fusion toxin would be able to bind to the GM-CSF receptor, resulting in the internalization of the GM-CSF receptor-fusion toxin complex into the endocytic vesicles in a fashion analogous to diphtheria toxin itself.[24,42] Our findings support this.

One major problem in myeloid leukemia is purging occult leukemia cells from patient BM before autologous BM transplant. Complete remission can be induced by intensive chemotherapy, but remission can be short-lived and the patient frequently experiences relapse of their underlying disease. Autologous BM transplantation is being explored as treatment modality designed to improve relapse-free survival. The patient's own BM is removed and then returned as a hematopoietic rescue procedure after aggressive chemotherapy and irradiation therapy. Because the procedure is complicated by metastatic leukemia cells infiltrating the BM, one

of the most commonly used techniques involve chemical purging of the BM to eliminate leukemia cells.[43] However, one drawback is that clinical chemical purging has a broad spectrum of depletion and eliminates beneficial cells including lymphocytes.[44] An advantage of using mGM-CSF to direct toxin is that GM-CSF would bind to myeloid leukemia cells[45] and not to cells that do not express the GM-CSF receptor.

Because residual metastatic leukemia cells that survive the preparative regimen can also lead to relapse and transplantation failure, DT_{390} mGM-CSF could be used for in vivo therapy, especially because there is clinical precedence for the use of DT-based fusion toxins for therapy of leukemia.[46] Although the in vivo efficacy of this agent will depend on its ability to access leukemia cells, their are several complicating issues that must also be explored. These issues can best be studied, and in some cases can only be studied, in animal models.

The issues are as follows: (1) In vivo depletion of myeloid cells might result in immunosuppression. Cells of myeloid origin participate as antigen presenting cells in generating optimal T-cell responses.[47] A fusion toxin directed against these cells might either reduce the immune response to tumor or render the host susceptible to secondary infections. (2) GM-CSF receptor expression on stem cells might limit the in vivo antileukemia effectiveness of DT_{390} mGM-CSF resulting in life-threating myelosuppression. The GM-CSF receptor is expressed on committed murine progenitor cells.[48] In fact, studies in humans show that GM-CSF is active in stimulating $CD34^+$ human progenitor cells.[49] In this paper, DT_{390} mGM-CSF was reactive against committed myeloid BM progenitors in in vitro CFU-GM assays. In separate studies (data not shown), we found that the fusion toxin had little effect against day 8 CFU-S, which measures earlier erythroid and myeloid stem cells, and day 12 CFU-S, which measures multilineage progenitor cells.[50,51] Thus, expression of mGM-CSF receptors occurs between CFU-S and CFU-GM stages of development. In future studies, DT_{390} mGM-CSF can be used in murine in vivo adoptive transfer experiments designed to directly determine stem cell expression of the GM-CSF receptor. Such experiments cannot be performed in humans. (3) Some believe that GM-CSF expression on endothelial cells could result in fusion toxin injury to the vasculature causing vascular leak sydrome, which has been problematic in the use of other immunotoxins.[52] GM-CSF stimulates the proliferation of endothelial cells.[53] However, our histopathologic studies of mice given in vivo DT_{390} mGM-CSF showed endothelialitis in some larger vessels, but no evidence of endothelial cell destruction (data not shown). (4) GM-CSF receptors are heterogenously expressed which might effect the efficacy of DT_{390} mGM-CSF against leukemic targets in vivo. Myeloid cells in mice appear to express two distinct types of GM-CSF receptors with high and low affinities, with kd of 20 to 60 pmol/L and 700 to 1,200 pmol/L, respectively.[40] The onset of proliferation of GM progenitor cells remains highly asynchronous, which may result from this heterogeneous expression of either high or low affinity GM-CSF receptors.

In conclusion, these findings describe a new fusion protein with high potential to eradiacate tumor cells of myeloid lineage. There is potential for using this agent to purge BM contaminated with leukemia or against minimal residual disease surviving current clinical conditioning regimens and causing relapse. The agent can be used to study the role of GM-CSF receptor expressing cells in lymphohematopoiesis. It is also noteworthy that the potential of DT_{390} mGM-CSF is not limited to myeloid leukemia because high-affinity GM-CSF receptors have been detected on solid tumor cell lines including those of lung cell or colon origin.[14-17] Thus, these tumors also may be effectively targeted.

ACKNOWLEDGMENT

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Granulocyte-Macrophage Colony-Stimulating Factor Receptor-Targeted Therapy of Chemotherapy- and Radiation-Resistant Human Myeloid Leukemias

JOHN P. PERENTESIS*,†, ANNE E. BENDEL, YU SHAO, BERTA WARMAN, STELLA M. DAVIES, CHANG H. YANG, MRIDULA CHANDAN-LANGLIE, KEVIN G. WADDICK and FATIH M. UCKUN

Biotherapy Institute and Departments of Pediatrics, Therapeutic Radiology-Radiation Oncology, Biochemistry, Pharmacology, University of Minnesota Academic Health Center, Minneapolis, Minnesota, USA

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Contemporary therapies for acute myeloid leukemia (AML) commonly fail to cure patients because of the emergence of drug resistance. Drug resistance in AML is multifactorial but can be associated with the overexpression of transmembrane transporter molecules, including P-glycoprotein (Pgp) or the multidrug resistance-associated protein (MRP), or associated with inactivation of the p53 tumor suppressor gene, as well as overexpression of the anti-apoptotic protein bcl-2. We are investigating if novel recombinant biotherapeutics can circumvent these resistance mechanisms to effectively treat refractory AML. To target the lethal action of diphtheria toxin (DT) to high affinity granulocyte-macrophage colony-stimulating factor (GMCSF) receptors on AML blasts, we have produced a recombinant chimeric fusion toxin, DT_{ct}GMCSF. Since DT_{ct}GMCSF enters and kills its target cells by unique mechanisms (GMCSF-receptor binding and protein synthesis inhibition) and is not similar in structure to Pgp or MRP substrates, we postulated that it would be an active agent against therapy-resistant AML. DT_{ct}GMCSF was selectively cytotoxic (IC_{50} 1–10ng/ml) to GMCSF-receptor positive AML cells expressing the Pgp- or MRP-associated multi-drug resistant phenotypes, despite high level resistance to conventional chemotherapeutic agents. DT_{ct}GMCSF also efficiently killed AML cells deficient in p53 expression, as well as radiation-resistant AML cells and mixed lineage leukemia cells expressing high levels of bcl-2. In addition, DT_{ct}GMCSF killed >99% of primary leukemic progenitor cells from therapy-refractory AML patients under conditions that we have previously found to not adversely affect the proliferative capacity or differentiation of pluripotent normal hematopoietic progenitor cells. DT_{ct}GMCSF may prove useful in treating myeloid leukemias that are otherwise resistant to a wide range of conventional therapies.

Keywords: Drug resistance, myeloid leukemia, recombinant fusion toxin, diphtheria toxin

*Corresponding author. Tel.: (612) 627–1920. Fax: (612) 627–1928.
†Present address: University of Minnesota Biotherapy Institute, 2625 Patton Road, Roseville, MN, 55113, USA.

INTRODUCTION

Multiagent chemotherapy regimens fail to cure more than half of the patients with AML because of the emergence of dominant multidrug and radiation-resistant subclones of leukemia cells.[1] Myeloablative radiochemotherapy followed by allogeneic or autologous bone marrow transplantation are associated with considerable morbidity and mortality, and have effected only modest improvements in the overall survival of AML patients, underscoring the need for rational drug design-based therapies for AML.[2]

Chemotherapy drug and radiation-resistance in AML can result from a variety of different mechanisms. In leukemic blasts, the overexpression of transmembrane transporter molecules that export a variety of natural cytotoxic agents with different mechanisms of action produces the multidrug resistance (MDR) phenotype, and this mechanism been implicated as a potential major cause for treatment failure in AML.[3,4] Several transporter proteins have been individually implicated in production of the MDR phenotype, and include P-glycoprotein (Pgp or P-170),[5,6] multidrug resistance associated protein (MRP),[7-9] and the recently described lung resistance-related protein (LRP).[10] In AML, expression of Pgp has been suggested to be associated with higher rates of chemotherapy remission induction failures, shorter relapse free intervals, and increased rates of relapse.[11-16] Other investigators have demonstrated increased expression of MRP in the leukemic blasts of patients with relapsed AML,[17-18] suggesting that the MDR phenotype in AML has a multifactorial etiology.

Another recently recognized important mechanism of treatment resistance in cancer and leukemia is associated with resistance to the induction of the programmed cell death pathway, or apoptosis, after malignant cells have incurred damage from chemotherapy or radiation.[19] Failure of normal apoptotic mechanisms have been associated, in part, with the development of malignancy.[20] Cellular damage inflicted by ionizing radiation and chemotherapeutic drugs with diverse molecular targets has been recognized to engage cellular mechanisms that result in apoptotic cell death in human hematopoietic malignancies.[21-23] In many malignancies, drug and radiation-resistance related to the failure of induction of apoptotic pathways has been associated with mutations in cell cycle control elements, including loss of function of the p53 tumor suppressor gene, or overexpression of the anti-apoptotic protein bcl-2. A primary requirement for the efficacy of any new therapy for AML is the ability to overcome classical mechanisms of drug- and radiation-resistance, as well as circumventing the failure to engage apoptotic pathways. However, most new agents have failed to surmount these barriers because treatment-refractory AML is a heterogeneous disease.

We are developing targeted therapies for AML that utilize highly potent cytotoxic substances that hold potential to circumvent classical pathways of multidrug resistance or to overcome resistance to apoptosis. Protein toxins produced by plants and bacteria that inactivate cellular protein synthesis may provide an effective alternate mechanism to kill AML cells that are otherwise resistant to conventional radio-chemotherapy regimens. Diphtheria toxin (DT) serves as the prototype for the class of protein synthesis inhibiting toxins that kill cells by catalyzing the ADP-ribosylation of the essential protein synthesis cofactor, elongation factor 2 (EF-2). EF-2 that has been ADP-ribosylated by DT cannot productively interact with the ribosome, and the consequent irreversible inhibition of protein synthesis leads to cell death.[24] The profound toxicity of DT is related to its ability to act catalytically, and the entry of a single molecule of the toxin into the cytoplasm is sufficient to kill a human cell.[25] DT-mediated protein synthesis inhibition thus provides a unique pathway for the induction of cell death, and is a mechanism of cytotoxicity distinct from other antimetabolites, antitumor DNA intercalating antibiotics, DNA damaging agents, or other drugs used in contemporary AML therapies.

DT has significant and lethal nonspecific toxicity that precludes its direct in vivo use in the treatment of malignancy. However, Murphy and colleagues have demonstrated that genetic engineering can be

employed to replace the indiscriminate binding domain of DT with cytokines, to specifically redirect the toxin's lethal action to cancer cells bearing the respective cytokine receptors.[26–32] In AML, the defective regulation of expression of the genes for individual hematopoietic growth factors or their receptors, and the resultant pathological autocrine or paracrine stimulation of growth, is increasingly implicated as one of the critical events involved in the etiology and maintenance of large subsets of myeloid leukemias.[33] A variety of investigators have observed constitutive expression and growth-stimulatory autocrine secretion of GMCSF in AML,[34–36] and leukemic cells from a majority of patients with AML exhibit an autonomous growth pattern related to GMCSF autocrine or paracrine production and secretion.[37] It has been demonstrated that leukemic myeloblasts can be effectively recruited into S phase of the cell cycle *in vivo* in AML patients by the exogenous administration of GMCSF.[38] In addition, the clinical use of GMCSF has been observed to increase peripheral blood blast counts in some patients receiving GMCSF for myeloid lineage disorders, and myelodysplasia patients with a high initial proportion of blasts may be particularly at risk.[39–41] It has been estimated that approximately 70% of patients with AML possess blasts with evidence of autonomous growth related to the autocrine secretion of growth factors, particularly GMCSF.[37] Overall, these studies indicate that high affinity GMCSF receptors are present on a significant number of myeloid leukemias, and may be reasonable candidates for the targeting of toxins in experimental therapeutic applications. In addition, receptors for GMCSF may be found on some non-hematopoietic solid tumors;[42] and hematopoietic growth factors, including GMCSF, IL-3, and G-CSF, can function in the stimulation of the *in vitro* clonal growth of select solid tumor cell lines and fresh tumor cells.[43–45]

We have developed a recombinant fusion toxin, $DT_{ct}GMCSF$, to target diphtheria toxin to the human GMCSF receptor. (*Bendel et al., Leuk. Lymphoma,* in press) In $DT_{ct}GMCSF$, the indiscriminant binding domain of diphtheria toxin is genetically replaced with hGMCSF (Fig. 1). The polymerase chain reaction was used to amplify the portion of the DT gene encoding the entire DT ADP-ribosyltransferase catalytic domain and the DT membrane translocation domain consisting of the hydrophilic amphipathic domain along with the first three hydrophobic alpha helical transmembrane sequences. An intervening linker was inserted between the DT moiety and the GMCSF moiety to allow for better exposure of the N-terminal helixes of GMCSF, the site of receptor recognition. $DT_{ct}GMCSF$ is specifically toxic to myeloid leukemia cell lines bearing the high affinity hGMCSF receptor but has no effect on other hematopoietic cell lines that lack these receptors. These cytotoxic effects of $DT_{ct}GMCSF$ can be blocked by a large excess of

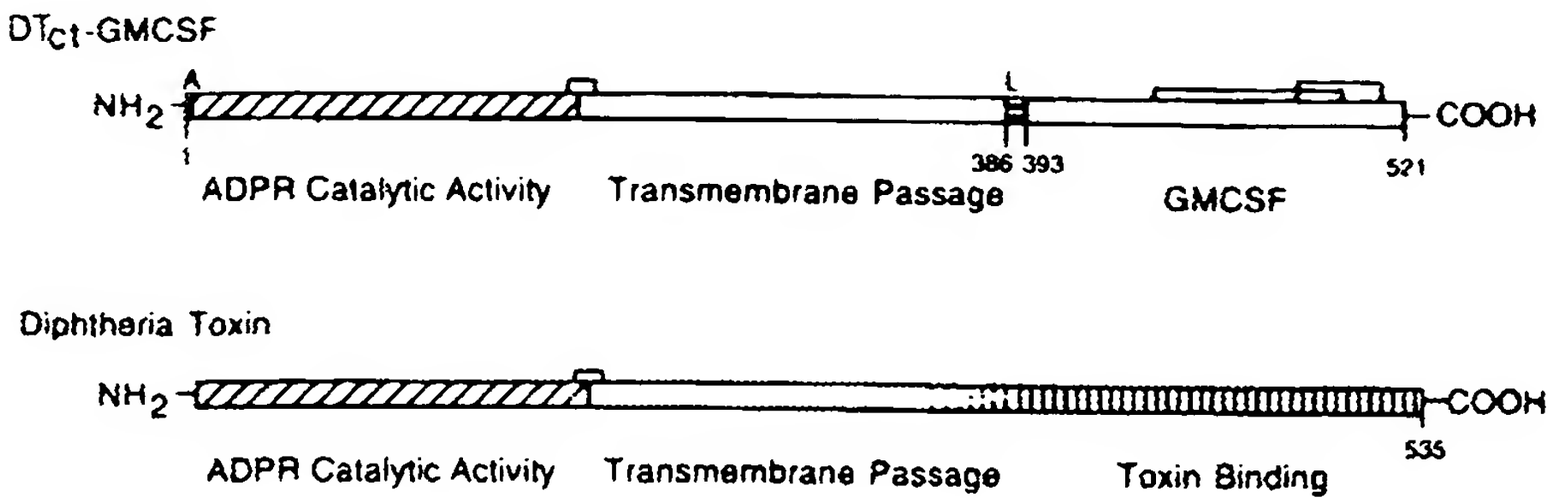

FIGURE 1 Structure of the recombinant $DT_{ct}GMCSF$ fusion toxin and comparision to diphtheria toxin. The native receptor binding domain of diphtheria toxin was genetically deleted and replaced with human GMCSF, separated by a short linking sequence ("L": Ser-$(Gly)_4$-Ser-Met). The genetic addition of an ATG codon was used to introduce a methionine residue to the amino terminus of the fusion toxin ("A" = Met).

hGMCSF, confirming that the cytotoxicity is mediated through the hGMCSF receptor.

In this study, we examined the ability of $DT_{ct}GMCSF$ to exert specific cytotoxicity against various leukemia cell lines that serve as models for the clinically important problems of drug and radiation resistance. We compared the activity of $DT_{ct}GMCSF$ with contemporary chemotherapeutic agents in the ability to kill these therapy-resistant cell lines. In addition, as an initial assessment of the potential clinical relevance of these observations, we evaluated the cytotoxic activity of $DT_{ct}GMCSF$ in leukemia progenitor cell assays using samples from therapy-refractory AML patients.

METHODS

Cell Lines and Culture Conditions GMCSF-R bearing human leukemia cell lines included the human acute promyelocytic leukemia cell line HL-60,[46] and the highly radiation-resistant mixed lineage acute leukemia cell line RS4;11[47] expressing high levels of bcl-2 protein.[48] These cell lines as well as the GMCSF-R negative control pre-B leukemia cell line NALM-6[49] were obtained from the American Type Culture Collection (Rockville, MD). Multidrug resistant subclones of HL-60 cells, including HL-60/VCR cells[50] which express a P-glycoprotein associated MDR phenotype, and HL-60/ADR cells[7,51] which express a MRP-associated MDR phenotype were the gift of Dr. M. Center (Kansas State University, Manhattan, KS). Cells were maintained in IMDM, 10% FBS and 50 U/ml penicillin, and 50 µg/ml streptomycin. Primary leukemic cells were obtained from the previously cryopreserved AML bone marrow samples of therapy-refractory patients stored in the liquid nitrogen tanks of the Children Cancer Group Cell Bank at the University of Minnesota.

Cytotoxicity Assays Cytotoxicity was measured by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assays.[52] For the MTT assays, cells were seeded into 96-well plates at a final concentration of 5×10^3 cells/well for TF-1, and $5–9 \times 10^4$ cells/well for HL-60, K562, NFS-60, MV4-11 and THP-1, and incubated at 37°C in a humidified incubator under a 5% CO_2 atmosphere for 16–24 hours. Dilutions of the $DT_{ct}GMCSF$ fusion toxin in PBS and 1% BSA or dilutions of vincristine in PBS/0.2% bovine serum albumin or doxorubicin in PBS/0.2% normal saline were added to each well and the incubation was continued for an additional 72–96 hours. MTT was added to a final concentration of 0.75 mg/ml with 4 hours of further incubation at 37°C. The dye was solubilized with 50% Isobutanol/10% SDS and cell viability was determined by measure of absorption (Å595) using a Bio-Rad Elisa Reader.

Clonogenic Leukemic Marrow Assays A serial dilution clonogenic assay system was used to evaluate the antileukemic efficacy of $DT_{ct}GMCSF$ against human leukemia cell lines, as previously described in detail.[53,54] In brief, approximately 1×10^6 cells/ml were incubated with $DT_{ct}GMCSF$ in RPMI 1640 medium supplemented with 20% heat-inactivated FCS at 37°C for 16 hours in a humidified atmosphere with 5% CO_2. After treatment, leukemia cells were washed twice in RPMI 1640, and a series of dilutions of the control and test cell suspensions were prepared in RPMI 1640 supplemented with 20%FCS, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 IU/ml penicillin, and 50 µg/ml streptomycin. A 100 µl volume of each dilution was plated in several wells of 96-well, flat-bottom tissue culture plates. Each well contained a monolayer of 1×10^5 irradited (500 cGy) bone marrow cells as a feeder layer. The wells were examined for clonogenic growth by using an inverted phase microscope after 14 days of culture at 37°C in a humidified atmosphere with 5% CO_2. Estimates of the most probable number of the remaining clonogenic units were made as previously described[55,56] using a modification of the Spearman-Karber method by counting the number of wells showing growth. The sensitivity of primary AML cells to $DT_{ct}GMCSF$ was examined in a leukemic progenitor cell assay system.[55,56]

Colony assays of normal bone marrow progenitor cells CFU-GEMM, CFU-GM, and BFU-E were previously described.[57] Bone marrow mononuclear cells from three healthy donors were kindly provided by Dr. J. McCullogh from the University of Minnesota Blood

Bank. The percent control recovery values were calculated using the formula: % Control recovery = [Mean number of colony forming units in $DT_{ct}GMCSF$ treated samples/Mean number of colony forming units in untreated samples] × 100.

RESULTS

GMCSF-R Specific Cytotoxic Activity of DT_{ct} GMCSF Against Myeloid Leukemia Cells Expressing the MDR Phenotype To test our hypothesis that $DT_{ct}GMCSF$ would not be susceptible to drug resistance mediated by overexpression of transmembrane transporter molecules, we examined the ability of it to kill chemotherapy-resistant HL-60 subclones. We evaluated the cytotoxicity of $DT_{ct}GMCSF$ against cells expressing the MDR phenotype, including HL-60/VCR cells which express a P-glycoprotein associated MDR phenotype, and HL-60/ADR cells which express an MRP-associated MDR phenotype. In assays of cellular viability, $DT_{ct}GMCSF$ was cytotoxic to both HL-60/VCR and HL-60/ADR cells (Fig. 2A). HL-60/ADR cells demonstrated an IC_{50} for $DT_{ct}GMCSF$ that was not significantly different from the IC_{50} of wild-type HL-60 cells, and the IC_{50} of HL-60/VCR cells was increased by less than an order of magnitude. Consistent with previous reports,[50,51] and in contrast to the results seen with $DT_{ct}GMCSF$, both HL-60/VCR and HL-60/ADR cell lines exhibited significant high-level resistance to cytotoxicity of the chemotherapeutic agents doxorubicin (Fig. 2B) and vincristine (Fig. 2C). Doxorubicin was required at over 100 fold higher concentrations to achieve an IC_{50} in either of the HL-60/VCR or HL-60/ADR cell lines, and vincristine was required at 50–100 fold higher concentrations to achieve an IC_{50} in either of the HL-60/VCR or HL-60/ADR cell lines.

$DT_{ct}GMCSF$ Clonogenic Killing of Primary Leukemic Blasts from Patients with AML We also used leukemic progenitor cell assays to examine the antileukemic activity of $DT_{ct}GMCSF$ against primary leukemic cells from 7 therapy-refractory AML patients. As shown in Table I, $DT_{ct}GMCSF$ killed 92–99.9% of leukemic progenitor cells from 5 of the 7 cases studied. The observed inhibition of blast colony formation was due to destruction of leukemic progenitor cells rather than an impairment in their ability to proliferate in methylcellulose cultures because cultures of $DT_{ct}GMCSF$—treated AML blasts did not contain microclusters (Fig. 3). These results provide direct evidence that $DT_{ct}GMCSF$ can kill clonogenic leukemia cells from a substantial proportion of AML patients who have failed conventional chemotherapy. Similarly, $DT_{ct}GMCSF$ effectively killed clonogenic cells of RS4;11 and HL-60 leukemia cell lines that were used as positive controls (Table I).

DISCUSSION

It is critical to discern if a new agent for AML therapy is effective in killing cells expressing the MDR phenotype, since drug resistance is one of the major reasons for the failure of contemporary AML treatment regimens. Since $DT_{ct}GMCSF$ enters and kills its target cells by unique mechanisms (GMCSF-R binding and protein synthesis inhibition, respectively), and is not apparently similar in structure to other P-glycoprotein or MRP substrates, we postulated that it would be an active agent against leukemia cells possessing the MDR phenotype. We found that overexpression of P-glycoprotein or MRP does not to cause a significant decrease in $DT_{ct}GMCSF$ cytotoxicity against human myeloid leukemia cells. The prominent role of the MDR phenotype in causing treatment failure and ultimate mortality in AML suggests that $DT_{ct}GMCSF$ may useful to complement the activity of conventional anti-neoplastic agents in the treatment of AML.

Mutation of the p53 gene and loss of its functional tumor suppressor activity is frequently observed in aggressive malignancies and associated with a failure of the induction of apoptotic cell death, and a poor response to conventional therapies.[58,59] In our studies, treatment of p53-deficient HL-60 leukemia cells with $DT_{ct}GMCSF$ resulted in the rapid and efficient induction of cell killing, suggesting that the fusion toxin could potentially circumvent chemotherapy and radiation resistance resulting from p53-deficiency.

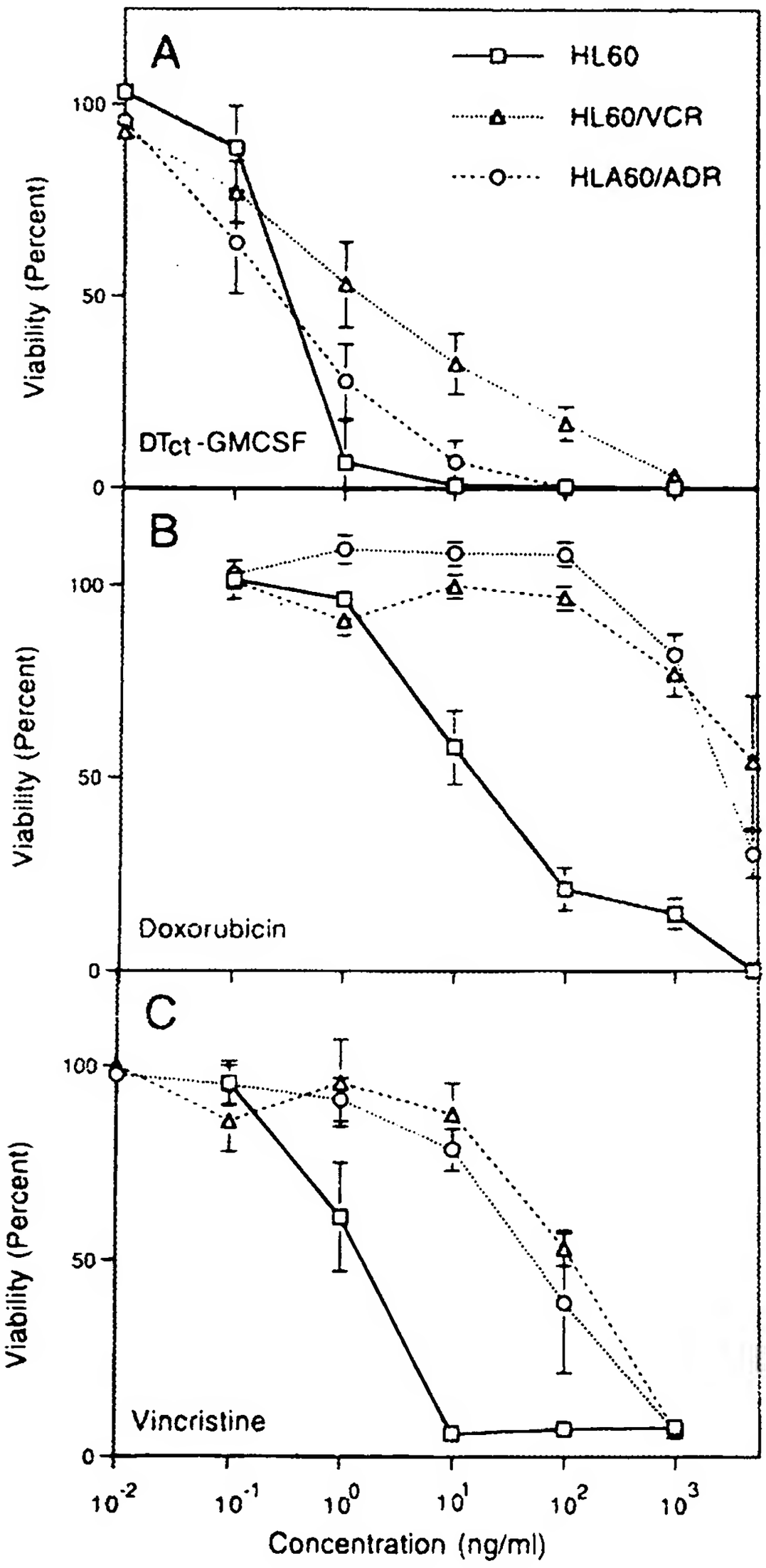

FIGURE 2 (A) $DT_{ct}GMCSF$ cytotoxicity to HL-60 and multidrug-resistant HL-60/VCR and HL-60/ADR cell lines, expressing P-glycoprotein and MRP, respectively. (B and C) Cytotoxicity of doxorubicin and vincristine, respectively, to HL-60 and multidrug-resistant HL-60/VCR and HL-60/ADR cell lines.

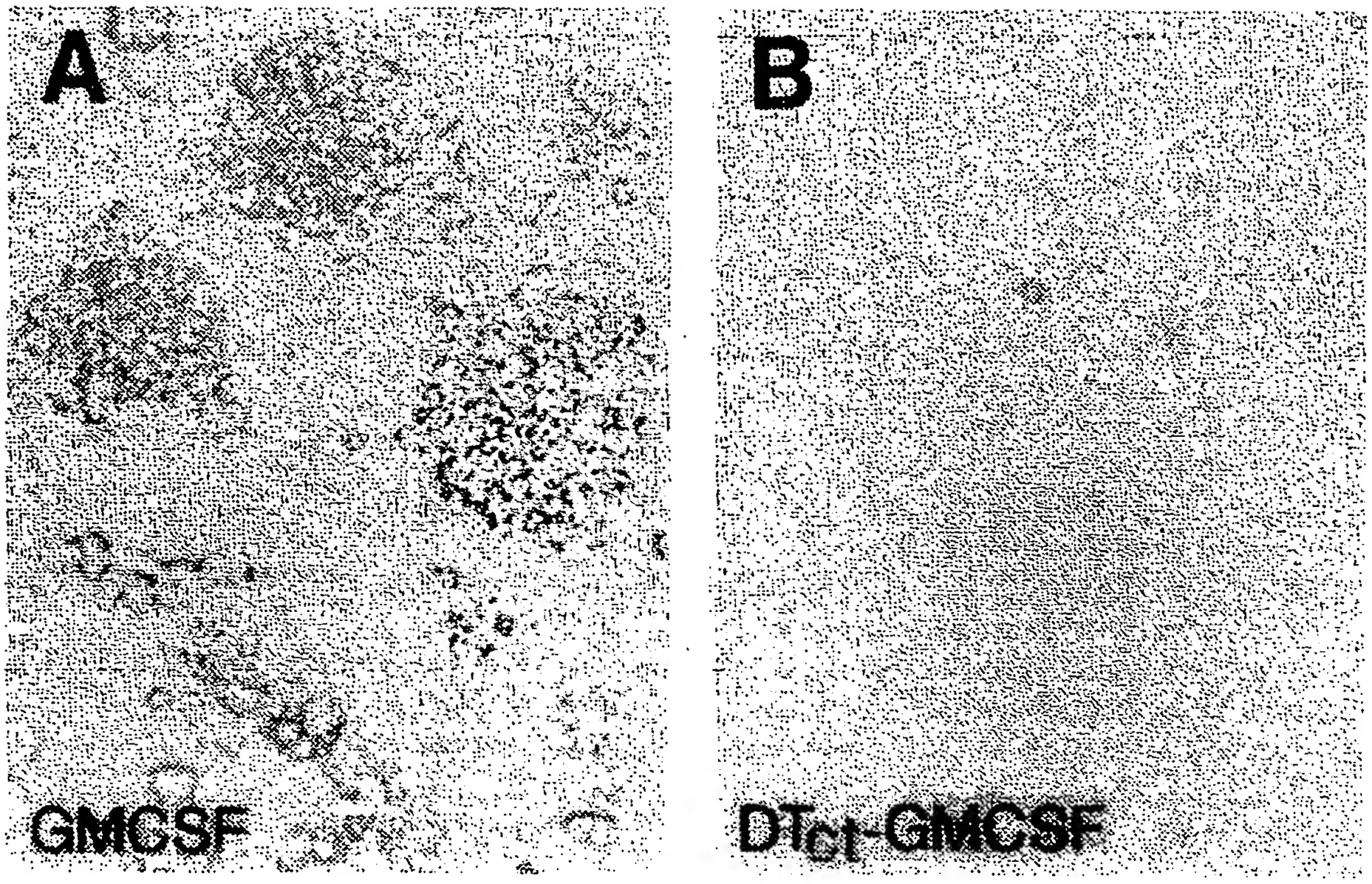

FIGURE 3 Leukemic progenitor cell-derived AML blast colony formation in in vitro cultures of GMCSF (1000 ng/mL)-treated (shown in A) vs DT_{α}GMCSF (1000 ng/mL)-treated (shown in B) primary leukemic cells from a therapy-refractory AML patient (Case #3 in Table 1). (See Color Plate XV at the back of this issue.)

TABLE I Effects of DT_{α} GMCSF on primary leukemic progenitor cells from therapy-refractory AML patients

Leukemic Progenitors		Mean No. Colonies/10^6 Cells (% Kill)		
		Control	DT_{α} GMCSF	
		1000ng/ml GMCSF	100 ng/ml	1000 ng/ml
AML	Case 1	1496 (–)	0 (>99.9)	0 (>99.9)
AML	Case 2	2204 (–)	1857 (15.7)	2390 (0)
AML	Case 3	90 (–)	0 (>98.8)	0 (>98.8)
AML	Case 4	642 (–)	512 (20.2)	338 (47.4)
AML	Case 5	19 (–)	1 (97.4)	0 (97.4)
AML	Case 6	13 (–)	0 (>92.3)	0 (>92.3)
AML	Case 7	41 (–)	0 (>97.6)	0 (>97.6)
AML	HL-60	785 (–)	4 (99.5)	4 (99.5)
t(4;11) ALL	RS4;11	3923 (–)	785 (80)	157 (96)

Chemotherapy resistance and failure of cell death induction has also been observed with the overexpression of the gene encoding bcl-2 or related proteins in malignant cell lines.[20] bcl-2 and related oncoproteins appear to function as central regulators in the prevention of apoptosis induction.[60] We observed that the DT_{ct}GMCSF cytotoxicity to HL-60 leukemia cells was not triggered by a decrease of their anti-apoptotic bcl-2 oncoprotein levels (data not shown). Indeed, DT_{ct}GMCSF effected destruction of radiation-resistant RS4;11 leukemia cells, providing evidence that high expression levels of bcl-2 oncoprotein associated with radiation resistance do not render GMCSF-R positive cells resistant to the potent cytotoxicity of the DT_{ct}GMCSF fusion toxin.

In addition, the activity of a new agent against the bulk population of leukemia cells does not always predict its activity against the clonogenic self-renewing subpopulations of leukemia cells. Importantly, DT_{ct}GMCSF was effective in the clonogenic killing of primary leukemia cells from patients with therapy-refractory AML.

Our data demonstrate that DT_{ct}GMCSF is a potent antineoplastic agent against GMCSF-R bearing leukemia cells. It effectively kills multidrug-resistant myeloid leukemia cell lines, leukemic cells deficient in p53 expression and leukemic cells expressing high levels of the bcl-2 oncoprotein. DT_{ct}GMCSF was also effective in killing primary leukemic progenitor cells from therapy-refractory AML patients, and might be useful in treating myeloid leukemias that are otherwise resistant to a wide range of conventional therapies.

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Modulation of the Apoptotic Response of Human Myeloid Leukemia Cells to a Diphtheria Toxin Granulocyte-Macrophage Colony-Stimulating Factor Fusion Protein

By Arthur E. Frankel, Philip D. Hall, Chris Burbage, Joseph Vesely, Mark Willingham, Kapil Bhalla, and Robert J. Kreitman

It has previously been shown that human granulocyte-macrophage colony-stimulating factor (GM-CSF) can be fused to a truncated diphtheria toxin (DT) to produce a recombinant fusion toxin that kills GM-CSF receptor–bearing cells. We now report that DT388–GM-CSF induces apoptosis and inhibition of colony formation in semisolid medium in receptor positive cells, and that the induction of apoptosis correlates with GM-CSF–receptor occupancy at low ligand concentrations. Also, the induction of apoptosis correlates with the inhibition of protein synthesis and is inversely related to the amount of intracellular antiapoptotic proteins (Bcl2 and $Bc1X_L$). Nine myeloid leukemia cells lines and four nonmyeloid leukemia cell lines were incubated with 0.7 nmol/L of ^{125}I–GM-CSF in the presence or absence of excess cold GM-CSF and bound label measured. High affinity receptor numbers varied from 0 to 291 molecules per cell. Cells were incubated with varying concentrations of recombinant fusion toxin for 48 hours and incorporation of ^{3}H-leucine (protein synthesis), segmentation of nuclei after DAPI staining (apoptosis), and colony formation in 0.2% agarose (clonogenicity) were measured. DT388–GM-CSF at 4×10^{-9} mol/L inhibited colony formation 1.5 to 3.0 logs for receptor positive cell lines. Protein synthesis and apoptosis IC_{50}s varied among cell lines from greater than 4×10^{-9} mol/L to 3×10^{-13} mol/L. GM-CSF–receptor occupancy at 0.7 nmol/L GM-CSF–ligand concentration correlated with the protein synthesis IC_{50}. Similarly, the protein synthesis inhibition and apoptosis induction correlated well, except in cells overexpressing Bcl2 and $BclX_L$, in which 25- to 150-fold inhibition of apoptosis was observed. We conclude that DT388–GM-CSF can kill acute myeloid leukemia blasts but that apoptotic sensitivities will depend on the presence of at least 100 high affinity GM-CSF receptors/cell and the absence of overexpressed antiapoptotic proteins.

ACUTE MYELOID leukemia (AML) was diagnosed in 9,200 patients in the United States in 1996,[1] and even with complete remission rates of 50% to 70% and effective consolidation and intensification regimens including allogeneic bone marrow transplantation, over 80% of patients will die from complications of the disease or its treatment.[2] Radiochemotherapy resistant blasts are a frequent cause of treatment failure in AML patients.[3] In many cases, blasts exhibit a multidrug-resistance phenotype produced, in part, by overexpression of drug efflux transporters (P-glycoprotein and multidrug-resistance protein) or overexpression of antiapoptotic peptides (Bcl2 and $BclX_L$). Prospective and retrospective clinical studies have shown a worse prognosis for patients with resistance phenotypes caused by high concentrations of these molecules.[4-7] Although nonspecific modulators of P-glycoprotein (quinine, cyclosporine, and PSC833) have been tested in AML therapy trials, to date these agents have been associated with significant toxicities to marrow and other organs, marked alterations of cytotoxic drug pharmacodynamics, and minimal effects on disease-free survival or overall survival.[8] Thus, there is a need for new reagents with unique mechanisms of action that can selectively modify the apoptotic threshold of malignant blasts.

One such novel class of AML therapeutics is targeted toxin molecules. These polypeptide drugs consist of myeloid leukemia–directed ligands covalently linked to protein synthesis inactivating peptide toxins. We chose to use diphtheria toxin as the toxophore and human granulocyte-macrophage colony-stimulating factor (GM-CSF) as the haptophore or ligand. Extensive experience both preclinically and clinically with diphtheria fusion toxins suggested this protein synthesis–inactivating peptide could efficiently kill malignant cells in vitro and in vivo.[9] The ligand GM-CSF was chosen because the receptor for this cytokine is present on the majority of AML patients' blasts.[10] The normal tissue distribution showed the presence of receptor on committed myeloid progenitor cells[11] and alveolar macrophages,[12] but not on primitive hematopoietic stem cells or other vital normal tissues.[13] We prepared DT388–GM-CSF by modification of diphtheria toxin cDNA.[14] Codons encoding DT amino acids 389 to 535 (the binding domain) were replaced with codons for human GM-CSF. We purified recombinant protein by refolding denatured and reduced inclusion body protein from *Escherichia coli* and showed potent selective protein synthesis inhibition of GM-CSF receptor–bearing cells. The fusion toxin intoxicates cells similarly to wild-type DT,[15-19] except for binding to the GM-CSF receptor. DT388–GM-CSF is similar to DTctGM-CSF, which contains diphtheria toxin–amino acid residues 1 to 385 fused to a Ser-$(Gly)_4$-Ser-Met linker to human GM-CSF.[20] Both recombinant toxins showed better in vitro potency in killing leukemia blasts than other anti-AML–targeted toxins, including anti-CD33 antibody coupled with chemically blocked ricin,[21] anti-CD33 antibody conjugated to gelonin,[22] and GM-CSF fused to genetically modified ricin or Pseudomonas exotoxin.[19,23] The greater efficiency of intoxication by the diphtheria fusion proteins may relate to their rapid escape to the cytosol from

From the Departments of Medicine, Pathology, and Pharmaceutical Sciences, Medical University of South Carolina, Charleston, SC; the Department of Medicine, Emory University School of Medicine, Atlanta, GA; and Laboratory of Molecular Biology, National Cancer Institute, National Institutes of Health, Bethesda, MD.

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Address reprint requests to Arthur E. Frankel, MD, Hollings Cancer Center Room 311, 86 Jonathan Lucas St, Charleston, SC 29401.

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the endosomal compartment. Ricin, Pseudomonas exotoxin, and presumably gelonin must avoid lysosomal routing and reach a post-Golgi compartment before translocation to the cytosol.[24]

The goal of the present study was to determine the quantitative ability of DT–GM-CSF to induce apoptosis in myeloid leukemia cell lines. Because the ultimate clinical efficacy of an AML therapeutic depends on modulating the apoptotic threshold of leukemic progenitors rather than solely inhibiting protein synthesis, we believed such a study was a requisite step in the clinical development of the drug.

MATERIALS AND METHODS

Characterization of DT388–GM-CSF. Protein, purified as described,[19] was quantitated by BioRad protein assay as per recommendations of the supplier (BioRad, Hercules, CA). Aliquots of DT388–GM-CSF and prestained low molecular weight protein standards (BioRad) were run on a reducing 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and either stained with Coomassie Blue R-250 (Sigma Chemical Co, St Louis, MO) or transferred to nitrocellulose, blocked with 10% Carnation nonfat dry milk/0.1% bovine serum albumen (BSA)/0.1% Tween 20, washed with phosphate-buffered saline (PBS) plus 0.05% Tween 20, reacted with 5 μg/mL rabbit anti–GM-CSF (Genzyme, Cambridge, MA), rewashed, incubated with alkaline phosphatase conjugated goat anti-rabbit IgG (Sigma), washed again, and developed with the Vectastain alkaline phosphatase kit (Vector Laboratories, Burlingame, CA) as per manufacturer's instructions. Gels and blots were scanned on an IBAS automatic image analysis system (Kontron, Germany).

DT388–GM-CSF binding affinity for the GM-CSF receptor was assayed by incubating 2×10^6 HL60 cells in 150 μL RPMI1640 plus 2.5% BSA (Sigma) plus 0.2% sodium azide (Sigma) plus 20 mmol/L HEPES (Sigma) pH 7.2 and different concentrations of yeast-derived GM-CSF (Immunex Corp, Seattle, WA) or DT388–GM-CSF. Finally, 5 μL containing 0.2 μCi radiolabeled ^{125}I–GM-CSF (114 μCi/μg, 43 μCi/mL; DuPont, Wilmington, DE) were added to each tube and the tubes incubated at 37°C for 1 hour. Samples were then overlaid on 200 μL of phthalate oil mixture (1.5 parts dibutylphthalate and 1 part octylphthalate; Aldrich Chemical, Milwaukee, WI), centrifuged for 1 minute at 10,000 rpm in a microcentrifuge, and cell pellets and supernatants counted in a LKB-Wallach 1260 multi-gamma counter gated for ^{125}I with 50% counting efficiency. Dissociation constant (kd) was calculated from Scatchard plots as the concentration of protein yielding 50% displacement of ^{125}I-ligand.

Cell culture. HL60 and HL60/VCR human leukemia cells were obtained from Dr A. Safa[25] and grown in RPMI1640 medium with 10% fetal bovine serum (FBS) and 100 U/mL penicillin and 100 μg/mL streptomycin. All media and components were purchased from Irvine Scientific (Santa Ana, CA). HL60/Bcl2, HL60neo, and HL60/BclX_L were isolated as previously described[26,27] and maintained in RPMI1640 plus glutamine with 10% FBS with 1 mg/mL G418 (Geneticin, Life Technologies, Grand Island, NY). U937, K562, CEM, KB, HEL92.1.7, and THP-1 human leukemia cells were obtained from the American Type Culture Collection (Rockville, MD) and maintained on RPMI1640 medium plus glutamine with 10% FBS and penicillin/streptomycin. TF1 cells were obtained from Dr R. Puri (National Institutes of Health, Bethesda, MD) and grown on RPMI1640 plus glutamine with 10% FBS, 50 ng/mL human GM-CSF (Immunex), 1 mmol/L sodium pyruvate, and 1× nonessential amino acids. KG-1 and AML193 cells were obtained from the American Type Culture Collection. Both cell lines were grown in Iscove's modified Dulbecco's medium with 25 mmol/L HEPES and 10% FBS. The AML193 cells were supplemented with 50 ng/mL GM-CSF. The properties of the cell lines and their sources are described in Table 1.

Table 1. Properties of Assayed Human Cell Lines

Cell Line	Description	GM-CSF Dependence	Reference
HL60	Acute myelogenous leukemia	–	25
HL60neo	HL60 with neo resistance	–	26
HL60/VCR	HL60 resistant to vincristine	–	25
HL60/Bcl2	HL60 with Bcl2 transgene	–	26
HL60/BclX_L	HL60 with BclX_L transgene	–	27
K562	Chronic myelogenous leukemia	–	48
KB	Mouth epidermoid carcinoma	–	49
CEM	T-lymphoblastic leukemia	–	50
HEL92.1.7	Erythroleukemia	–	51
TF1	Erythroleukemia	+	52
U937	Monocytic leukemia	–	53
THP-1	Acute monocytic leukemia	–	54
KG-1	Acute myelogenous leukemia	–	55
AML193	Acute monocytic leukemia	+	56
LS174T	Colon carcinoma	–	57

GM-CSF–receptor density. One to 12 million cells in RPMI1640 plus 2.5% BSA plus 0.2% sodium azide plus 20 mmol/L HEPES pH 7.2 in a total volume of 150 μL were incubated with 5 μL of ^{125}I–GM-CSF (114 μCi/μg, 43 μCi/mL) in the presence or absence of 1,500 ng unlabeled human GM-CSF for 1 hour at 37°C. The cell suspensions were then layered on 200-μL phthalate oil mixture (1.5 parts dibutylphthalate and 1 part dioctylphthalate) in 1.5 mL Eppendorf tubes, centrifuged at 10,000 rpm in a microfuge at room temperature for 1 minute, and the cell pellets counted in gamma counter with 50% efficiency as above. Counts per minute bound in the presence of cold GM-CSF were subtracted from total counts per minute and receptor occupancy calculated based on 4×10^{-6} cpm/bound molecule and the total number of cells. Because 2 ng of labeled GM-CSF representing 1×10^{-13} moles was diluted in 150 μL, the ligand concentration was 7×10^{-10} mol/L and the kd for GM-CSF on HL60 receptors was 1×10^{-10} mol/L,[23] liquid concentration was sevenfold above the 50% dissociation concentration for high affinity receptors. Further, using an average of 5×10^6 cells and 200 receptors/cell, a 60-fold excess of ligand over receptor was obtained. Increasing the labeled ligand concentration yields higher receptor occupancy for low affinity receptors (kd = 1 to 2 $\times 10^{-9}$ mol/L).[19]

Bcl2 content of cells. Thirty-five–millimeter Costar dishes were incubated with 2 mL of 1-mg/mL polylysine (Sigma) in PBS for 15 minutes at 37°C then rinsed three times with PBS. Two hundred thousand cells in 2 mL serum-free medium were then placed in the dishes and centrifuged at 2,400 rpm for 10 minutes in a low-speed centrifuge without a brake. The media was discarded and the dishes washed three times with PBS and fixed with 3.7% formaldehyde (10% formalin) in PBS for 10 minutes at room temperature. The dishes were rinsed again with PBS and blocked with 1% BSA (Sigma)/0.1% saponin (Sigma) in PBS for 15 minutes. All reactions were performed at room temperature. The blocking solution was removed and monoclonal antibody 124 anti-Bcl2 (Dako, Carpinteria, CA) at 10 μg/mL in 1% BSA/.01% saponin/PBS was reacted with the cells for 30 minutes. Again the cells were rinsed three times with PBS, and goat anti-mouse Ig conjugated to rhodamine (Jackson ImmunoResearch, West Grove, PA) was incubated with the cells for 30 minutes. The dishes were rinsed again three times with PBS and postfixed 10 minutes with 3.7% formaldehyde in PBS and

mounted in glycerol to PBS (90:10) and examined using a Zeiss Axioplan epifluorescence microscope.

Protein synthesis inhibition assays. Twelve different concentrations of DT388–GM-CSF (4×10^{-9} mol/L to 1.7×10^{-13} mol/L) were incubated with 1.5×10^4 cells in Costar 96 well flat-bottomed plates in a total volume of 150 μL of media used for cell growth for 48 hours at 37°C/5% CO_2. All assays were performed in duplicate. Fifty microliters of media containing 1 μCi ^{3}H-leucine was then added to each well and incubation at 37°C/5% CO_2 continued for an additional 4 hours. Cells were then obtained on a Skatron Cell Harvestor onto glass fiber mats and ^{3}H counts per minute counted in an LKB liquid scintillation counter gated for ^{3}H. The calculated IC_{50} was the concentration of protein that inhibited protein synthesis by 50% compared with control wells without DT388–GM-CSF.

Apoptosis assays. Aliquots of 1×10^5 cells were incubated in 24-well Costar plates at 37°C/5% CO_2 for 48 hours in media with 10 different concentrations of DT388–GM-CSF. Cells were then bound to polylysine-coated 35-mm dishes as described above, fixed in methanol containing 1 μg/mL DAPI (Sigma) to label nuclear DNA, and the percent apoptotic cells was determined by evaluating nuclear morphology by epifluorescence microscopy. The IC_{50} was the concentration of protein-inducing apoptosis in 50% of cells. Measurement of nuclear fragmentation for apoptosis using DAPI staining has been previously described.[28]

Clonogenic assay. Aliquots of 1×10^5 cells were incubated in wells of Costar 24-well plates at 37°C/5% CO_2 in 1 mL RPMI 1640 plus 15% FCS for 48 hours with 10 different concentrations of DT388–GM-CSF. Cell aliquot (0.1 mL) containing 1×10^4 cells was then dispersed in 3.4 mL RPMI 1640 plus 15% FCS with 50 ng/mL interleukin-3 (IL-3; PharMingen), GM-CSF and G-CSF (Amgen), and 0.2% agarose (SeaPlaque; FMC BioProducts, Rockland, ME) in 35-mm gridded polystyrene petri dishes. After 20 minutes solidification at 4°C, dishes were incubated 7 to 12 days at 37°C/5% CO_2 in a humidified incubator. Colonies containing more than 20 cells were counted on an inverted microscope.

RESULTS

Our goal is to develop DT388–GM-CSF for therapy of drug-resistant AML. For the rational design of clinical trials, we must define the variables that affect sensitivity of AML blasts to the drug. In this study, we have examined the effects of cell surface GM-CSF–receptor density and intracellular concentrations of antiapoptotic proteins on the induction of apoptosis by DT388–GM-CSF.

DT388–GM-CSF properties. Purified recombinant protein assayed colorimetrically yielded 800 μg/mL. Densitometry of Coomassie-stained gels and immunoblots using anti–GM-CSF antibody showed a single detectable band at 60 kD molecular weight (Fig 1). The affinity of DT388–GM-CSF for GM-CSF receptor was 1.4×10^{-10} mol/L compared with 1.0×10^{-10} mol/L for GM-CSF alone (Fig 2).

Distribution of GM-CSF receptor on cell lines. We measured GM-CSF receptor occupancy at 0.7 nmol/L ligand by incubating cells with radiolabeled ligand and determining the bound number of molecules. Using this method, reproducible receptor occupancies were obtained (Table 2). For myeloid blasts requiring GM-CSF in their growth media (TF1 and AML193), prewashing with medium lacking growth factor was critical for accurate assay. Receptor occupancy varied from 0/cell for K562, CEM, and HEL92.1.7 to 291/cell for THP-1. Low affinity sites/cell for U937, HL60, and TF1 were reported to be 3,500, 540, and 3,700 sites/cell, respectively.[19]

Bcl2 content of cell lines. Background fluorescence intensity for Bcl2 detection was 5 mU from the Zeiss Axioplan photometer average of five single cells for HL60Bcl2 without primary antibody. HL60, HL60BclXL, TF1, and HEL92.1.7 yielded the same 5 mU of Bcl2 immunofluorescence. In contrast, HL60Bcl2 produced 16 mU and HL60/VCR showed 8 mU (Fig 3). The cell-to-cell variation in fluorescence intensity was less than 1 mU for all cell lines except HL60Bcl2, which showed a range of 11.7 to 18.8 mU with a standard deviation of 1.4 mU.

Protein synthesis inhibition of cell lines by DT388–GM-CSF. DT388–GM-CSF at 4×10^{-9} mol/L produced greater than 90% inhibition of protein synthesis of all cell lines except CEM, HEL92.1.7, K562, and LS174T (Fig 4 and Table 3). The IC_{50}s varied for the remaining cell lines varied from 3×10^{-10} mol/L for AML193 to 3×10^{-13} mol/L for U937. Linear regression analysis showed correlation between high affinity GM-CSF–receptor density (receptor occupancy at 0.7 nmol/L) and protein synthesis–inhibition sensitivity to DT388–GM-CSF (Fig 5, $r^2 = .56$).

Apoptosis induction of cell lines by DT388–GM-CSF. All DT388–GM-CSF–sensitive cell lines showed morphological evidence of apoptotic nuclear segmentation (Fig 6). Concentrations of DT388–GM-CSF produced 50% apoptosis after 48 hours incubation (IC_{50}) as shown in Table 4. IC_{50} for apoptosis correlated well with IC_{50} for protein synthesis inhibition (ratios, 1 to 3), except for the three cell lines with overexpression of Bcl2 (HL60Bcl2 and HL60/VCR) or $BclX_L$ (HL60$BclX_L$). In these three cell lines, much higher concentrations of DT388–GM-CSF (25- to 150-fold) were required to achieve the same degree of apoptosis.

Inhibition of colony formation by DT388–GM-CSF. Each of the DT–GM-CSF–sensitive cell lines showed inhibition of colony formation in agarose (Fig 7 and Table 5). Maximal concentrations of DT388–GM-CSF (4×10^{-9} mol/L) yielded 1.5 to 3.0 log reduction in colony number in each case. The IC_{50}s for colony inhibition were similar to those for apoptosis and protein synthesis inhibition.

DISCUSSION

The bacteria-derived recombinant fusion toxin DT388–GM-CSF was soluble, had the molecular weight predicted for the 515 amino acid sequence, and showed immunoreactivity with anti–GM-CSF antibody. Gel electrophoresis showed the protein purity was greater than or equal to 95%. The DT388–GM-CSF had better affinity for the GM-CSF receptor than GM-CSF–ricin by 1.8-fold.[23] The crystal structure of GMCSF,[29] mutational analysis,[30] and blocking monoclonal antibody studies[31] previously showed that the cytokine is a member of the four helix bundle family and the ligand interacts with receptor with the principal amino acid residues clustered midway along helices A and C and with some residues at the C-terminus. Thus, attachment of GM-CSF to the C-terminus of DT avoiding steric blockage of both the middle and C-terminus of GM-CSF would be expected to yield the best affinity with receptor. Further, x-ray crystal structure of DT shows amino acid residues 380 to 388 are

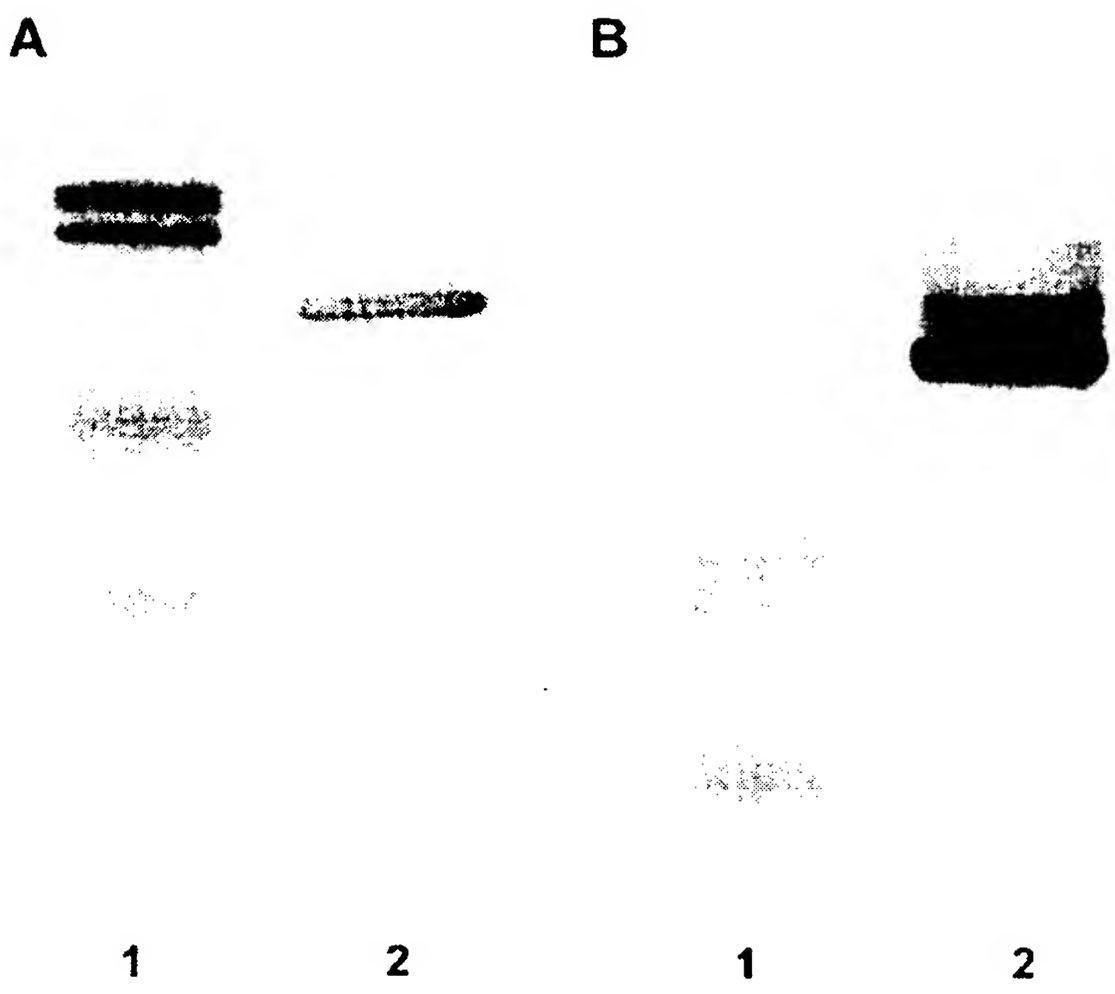

Fig 1. Fifteen percent reducing SDS-PAGE. (A) Coomassie stained. (B) Immunoblot reacted with rabbit anti-human GM-CSF antibody. Lanes in (A) and (B): lane 1, low molecular weight prestained Bi-oRad protein standards with molecular weights 101 kD, 83 kD, 51 kD, 36 kD, and 29 kD; lane 2, DT388–GM-CSF.

separate from the translocation domain and permit extensive freedom of movement for the C-terminal domain.[16] This linker sequence should provide more flexibility than the ADP tripeptide used to link GM-CSF and ricin.

The assay we used to quantitate GM-CSF receptor density used an intermediate concentration of radiolabeled GM-CSF that was sevenfold higher than the kd of the high affinity receptor and twofold less than the kd of the low affinity receptor.[32] Thus, we measured the high affinity receptors (ligand occupancy of more than 90% for high affinity receptor) and a fraction (<20%) of the low affinity receptors. The high affinity receptors have been shown to be heterodimers of α and β subunits, whereas the low affinity receptors consist of α subunits alone.[33] The low to absent number of high affinity receptors on non-AML cell lines agrees with previous observations.[34-35] The presence of high affinity receptors on AML cell lines that are not dependent on exogenous GM-CSF has been previously documented[10] and may be caused by autocrine production of GM-CSF[36] or hyperphosphorylation of raf-1, an intermediate in the GM-CSF signal-transduction pathway.[37]

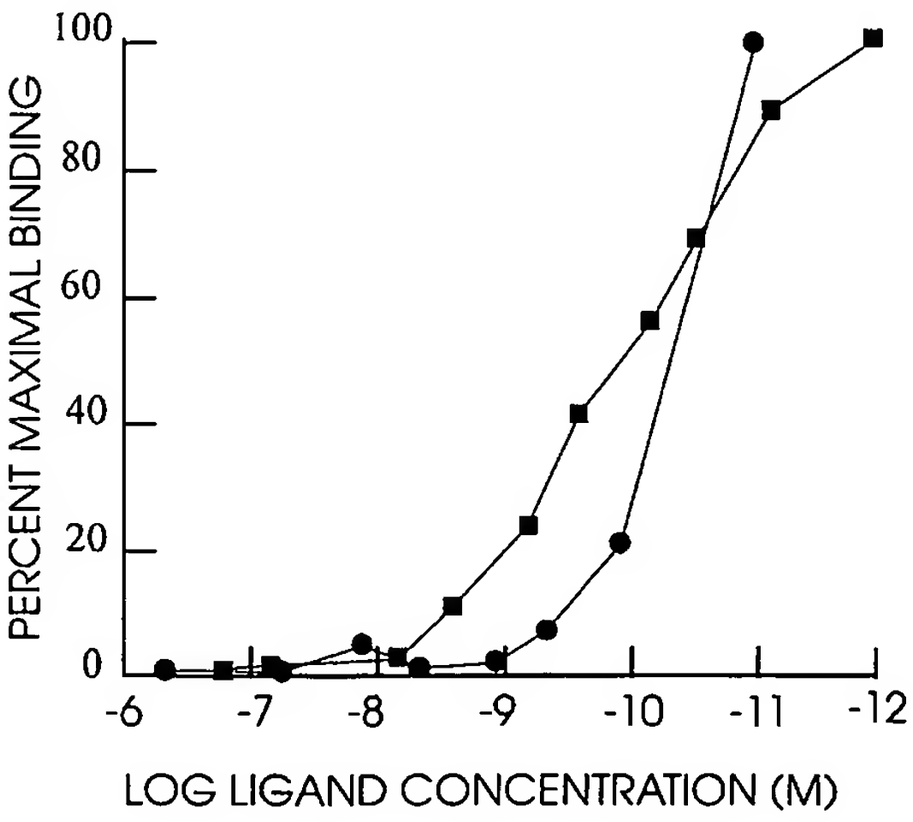

Fig 2. Competition binding experiment with HL60 cells. A total of 2×10^6 cells mixed with different concentrations of human GM-CSF or DT388–GM-CSF and 0.2 μCi ^{125}I–labeled GM-CSF in 150 μL RPMI1640 with 2.5% BSA and 0.2% sodium azide and 20 mmol/L HEPES pH 7.2 and incubated for 1 hour at 37°C. Cells were centrifuged through a phthalate oil mixture and free and bound counts per minute determined. Scatchard analysis was used to determine kds.

Table 2. GM-CSF–Receptor Occupancy at 0.7 nm Ligand for Assayed Cell Lines

Cell Line	Receptors Occupied/Cell
THP-1	291
KB	272
HL60neo	195
U937	178
TF1	165
HL60/VCR	158
HL60	156
HL60Bcl2	156
KG1	136
HL60BclXL	119
AML193	80
LS174T	42
HEL92.1.7	0
K562	0
CEM	0

Assay performed in duplicate as described in text. Values are means.

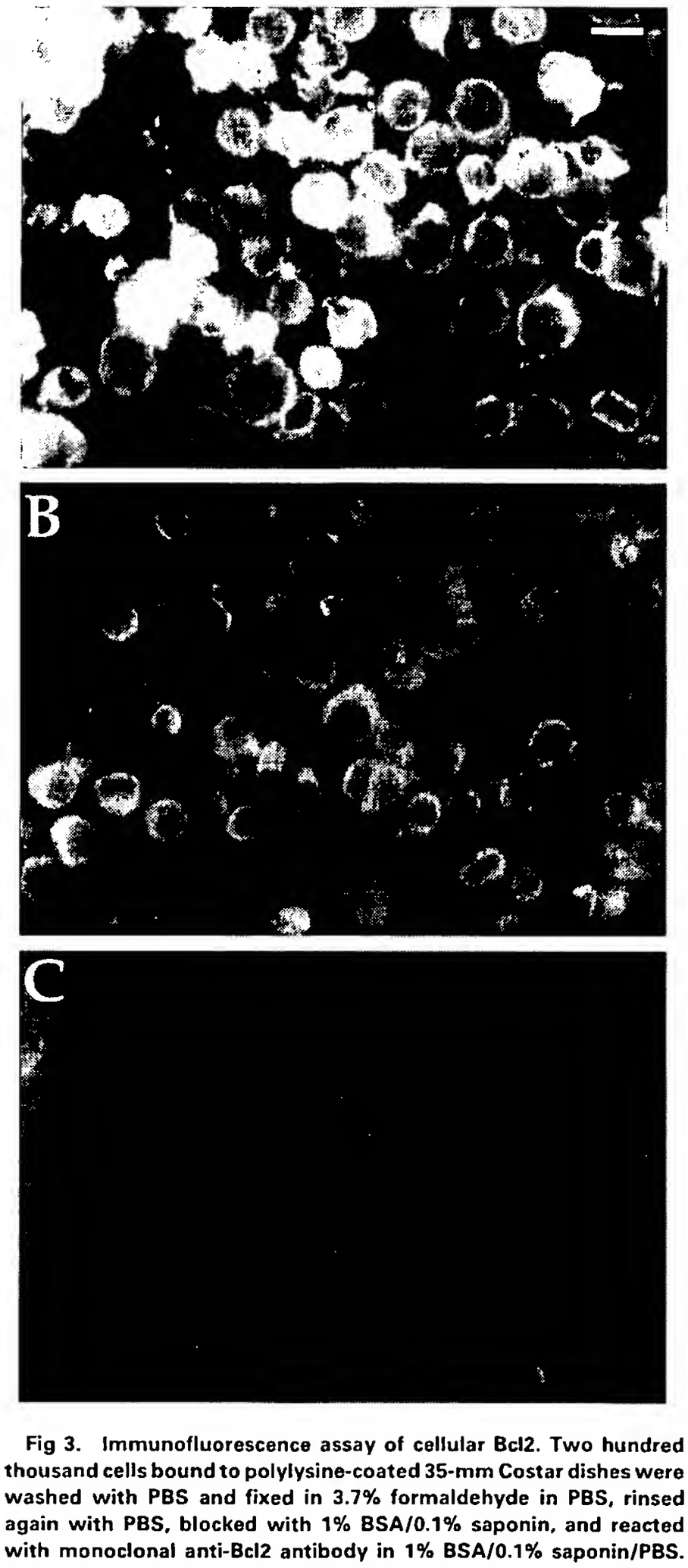

Fig 3. Immunofluorescence assay of cellular Bcl2. Two hundred thousand cells bound to polylysine-coated 35-mm Costar dishes were washed with PBS and fixed in 3.7% formaldehyde in PBS, rinsed again with PBS, blocked with 1% BSA/0.1% saponin, and reacted with monoclonal anti-Bcl2 antibody in 1% BSA/0.1% saponin/PBS. Cells were again rinsed with PBS and bound antibody detected with rhodamine conjugated goat anti-mouse Ig. After a final rinse with PBS and postfixing in 3.7% formaldehyde, dishes were mounted in glycerol:PBS and examined under a Zeiss Axioplan epifluorescence microscope. Magnification is × 350; scale bar represents 17 μm. (A) HL60; (B) HL60/VCR; (C) HL60BclX_L.

Bcl2 measurements yielded a positive signal on HL60/VCR cells not previously reported. Because these cells were selected by chronic exposure to 1 μg/mL vincristine,[25] they may overexpress a number of multidrug-resistance proteins such as bcl2, in addition to their known overexpression of

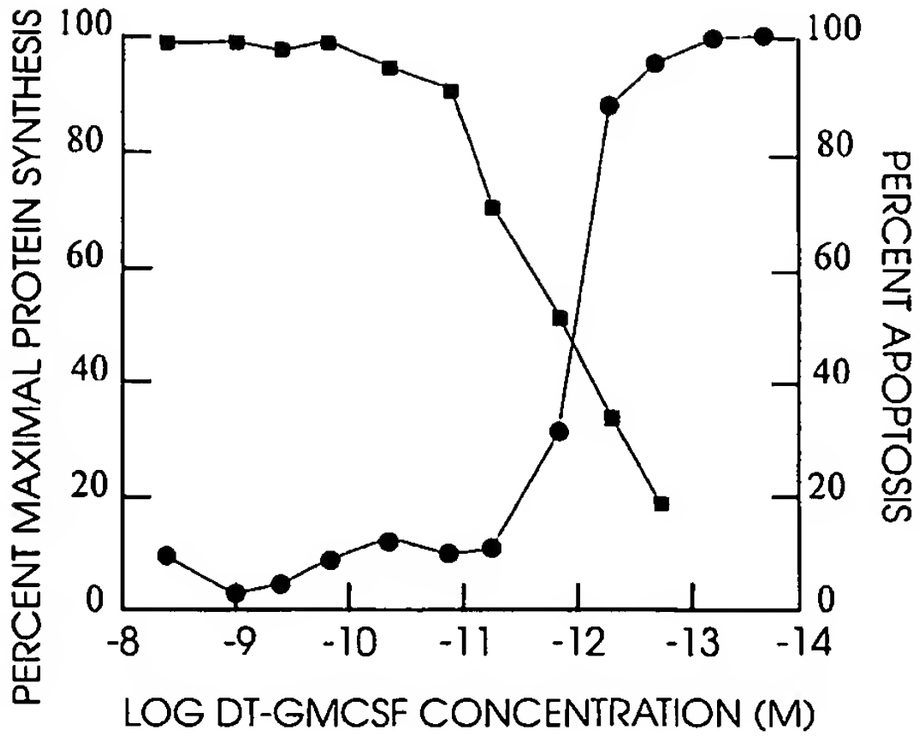

Fig 4. Cell cytotoxicity of DT388–GM-CSF on TF1 human leukemia cells. Cells were exposed at the dilutions indicated for 48 hours at 37°C/5% CO_2. Incorporation of ^{3}H-leucine was assayed after an additional 4 hours incubation and compared against untreated cell incorporation. The percent apoptotic nuclei based on DAPI staining were determined after 48 hours for different dilutions of DT388-GMCSF. (●) % maximal protein synthesis; (■) % apoptosis.

P-glycoprotein. A similar selection process showed overexpression of a number of resistance genes in MCF7 cells grown in the presence of adriamycin.[38]

Protein synthesis inhibition by DT388–GM-CSF was observed only for cell lines showing greater than 100 high affinity receptors/cell. Dependence of fusion toxin sensitivity on expression of high affinity receptors has been documented for DAB_{389}IL-2.[39] Because internalization is a prerequisite for DT intoxication,[18] and only high affinity α/β-receptors will mediate receptor-mediated endocytosis,[40] these results are consistent with molecular models of DT fusion protein action. The low number of receptors necessary for effective

Table 3. Sensitivity of Cell Lines to Protein Synthesis Inhibition by DT388–GM-CSF

Cell Line	IC_{50} ($\times 10^{-13}$ mol/L)
U937	3
HL60BclX_L	10
TF1	10
HL60neo	15
HL60Bcl2	20
HL60	50
THP-1	70
KG1	80
KB	100
HL60/VCR	120
AML193	3,000
K562	3,000
CEM	20,000
HEL92.1.7	>40,000
LS174T	>40,000

Assays performed in triplicate and mean values displayed as described in text.

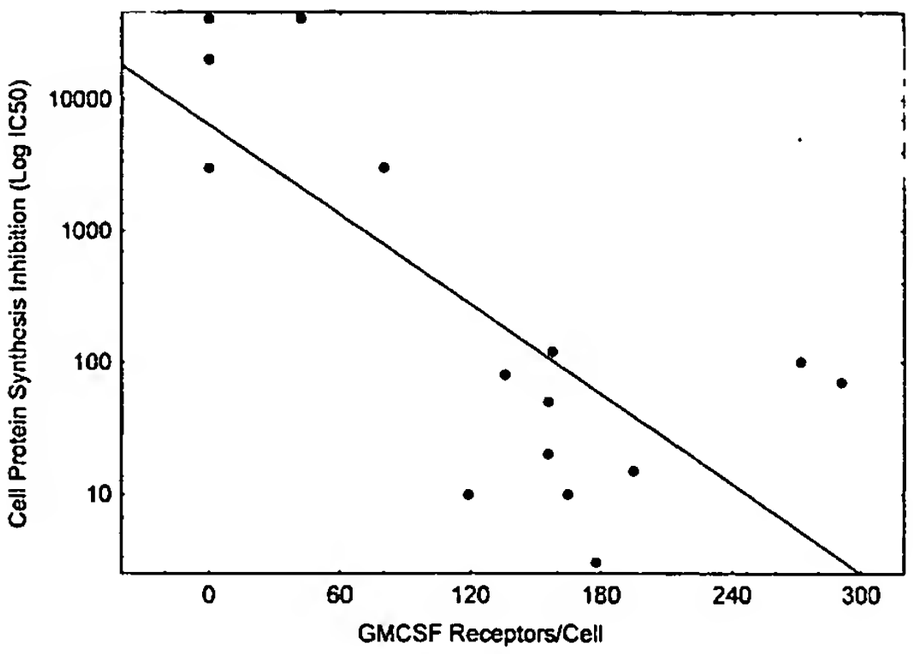

Fig 5. Plot of GMCSF receptors/cell (measured at ligand concentration of 0.7 nmol/L) versus IC_{50} for DT388–GM-CSF (concentration of drug inhibiting cell protein synthesis by 50%). Linear regression performed yielded log (IC_{50}) = 0.11*(GM-CSF receptor occupancy at 0.7 nmol/L) + 3.806 with r^2 = .557.

DT intoxication has been previously observed for DAB_{389}IL-2 and native DT.[17,39] Most normal tissues, except for normal myeloid progenitors,[13] pulmonary alveolar macrophages,[12] and possibly some endothelial cells,[41] lack high affinity GM-CSF receptors. Systemic administration of DT388–GM-CSF should produce transient myelosuppression, may affect pulmonary antimicrobial defenses, and may yield tissue-specific vascular leak. Upregulation of high affinity GM-CSF receptors on leukemic blasts may be possible with infusions of interferon-γ.[42]

Table 4. Apoptotic Threshold for Cell Lines Exposed to DT388–GM-CSF

Cell Line	IC_{50} (1×10^{-13} mol/L)	Ratio PSI IC_{50}/Apopt.IC_{50}
U937	10	3
TF1	20	2
HL60neo	50	3
HL60	50	1
KG1	80	1
HL60Bcl2	500	25
THP-1	240	3.5
HL60BclX_L	1,500	150
HL60/VCR	4,000	30
AML193	4,000	1.3
CEM	>40,000	ND
HEL92.1.7	>40,000	ND

Assays performed as described in text on at least two separate times. The cell line data were divided into two groups: those with and without elevated anti-apoptotic protein concentrations. A contingency table was then established using ratios less than 4 and greater than or equal to 4. Using Fisher's exact test the probability of a result as extreme as observed under the null hypothesis that elevated and nonelevated ratios come from the same population is less than 0.01.

Abbreviation: ND, not determined.

Induction of apoptosis by DT–GM-CSF has been reported for sensitive and drug resistant AML cells.[20] However, quantitative sensitivities to apoptosis induction have not been previously reported. The current study showed a close correlation between protein synthesis inhibition and apoptotic threshold, except for cells overexpressing the antiapoptotic

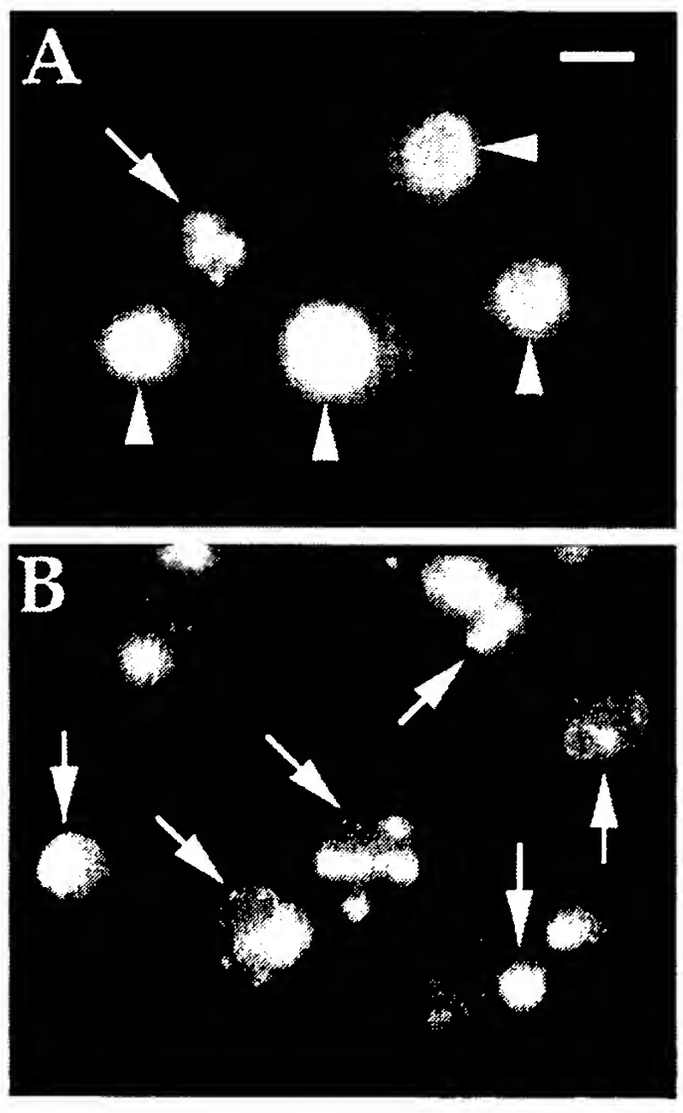

Fig 6. DAPI-stained cells treated with DT388–GM-CSF as described in text. (A) K562 cells (one apoptotic cell); (B) U937 cells (all apoptotic cells). Magnification is × 600; scale bar represents 10 μm; arrows indicate apoptotic nuclei; arrowheads indicate normal nuclei.

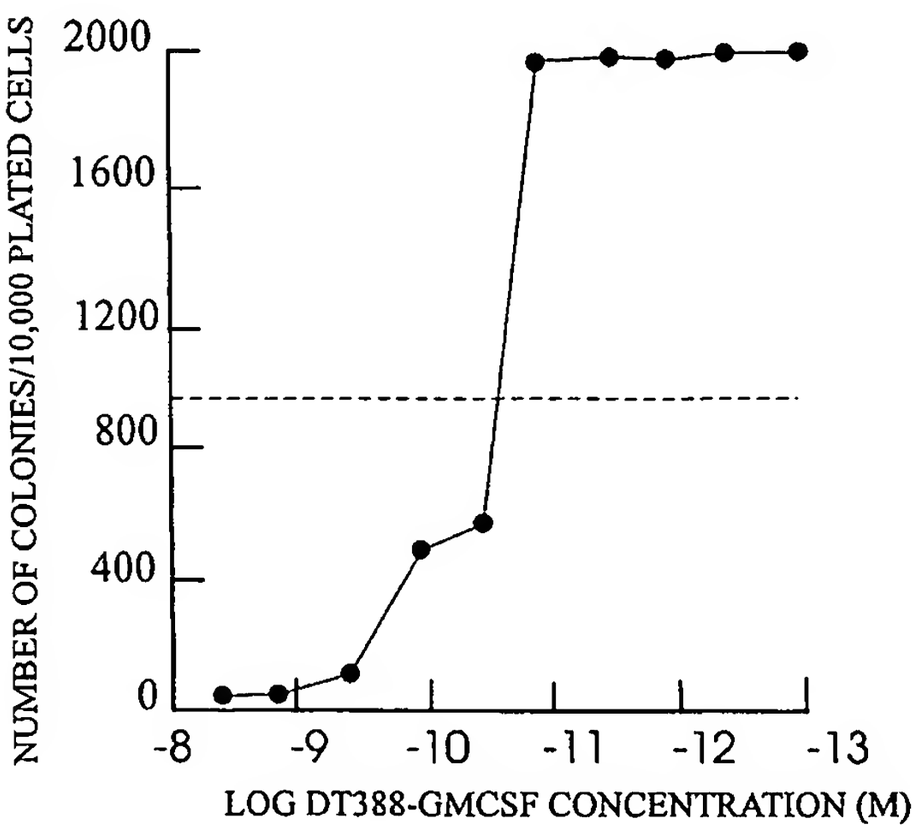

Fig 7. Colony formation inhibition by DT388–GM-CSF as described in text. Number of colonies with greater than 50 cells at 14 days from 10^4 cells shown on Y-axis. DT388–GM-CSF concentration for 48 hour preincubation shown on X-axis.

Table 5. Colony Formation for Cell Lines Reacted With DT388–GM-CSF

Cell Line	Maximal Log Cell Kill	IC_{50} ($\times 10^{-12}$ mol/L)
U937	3.5	1
HL60neo	2.0	10
AML193	2.5	10
HL60	3.0	20
KG1	2.0	20
THP-1	1.8	20
HL60BclX_L	1.5	40
HL60VCR	2.5	40
HL60Bcl2	2.6	40
K562	0	>4,000
CEM	0	>4,000
HEL92.1.7	0	>4,000

Assay performed as described in text at least twice.

proteins bcl2 and bclX_L. These results imply a critical role for reduction in new protein synthesis for triggering apoptosis and the use of this highly conserved (similar to the ced9 pathway in *Chlamydia elegans*) apoptosis pathway by diphtheria toxin.[43] Targeting the antiapoptotic peptide with antisense oligothionucleotides to Bcl2 or bclX_L may facilitate AML killing with DT388–GM-CSF.[44]

Clonogenic assays in semisolid medium have been used to assess progenitor blast viability[45] and was used in this work to confirm the observations of protein synthesis inhibition and apoptosis induction. The results show selective toxicity of DT388–GM-CSF for clonogenic receptor positive cell lines.

These results also have pertinence to the clinical development of DT–GM-CSF. In particular, patients with greater than 100 high affinity GM-CSF receptors/blast and with lack of expression of bcl2 and bclX_L will likely benefit from DT–GM-CSF therapy. However, extension from cell line studies to activity on fresh leukemic cells should be viewed with caution as, in several cases, targeted toxins perform better on cell lines than fresh leukemic cells.[46,47] Confirmatory clonogenic assays on fresh AML blasts will be needed to confirm the findings reported with established hematopoietic cell lines.

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RESISTANCE OF MYELOID LEUKAEMIA CELL LINES TO RICIN A-CHAIN IMMUNOTOXINS

ANDREAS ENGERT*, ALEX BROWN and PHILIP THORPE†

Drug Targeting Laboratory, Imperial Cancer Research Fund, Lincoln's Inn Fields, London WC2A 3PX, U.K.

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Abstract—Nineteen monoclonal antibodies that recognize antigens on myeloid leukaemia cells were screened upon HL60, KG1, U937 and K562 cells for their ability to form effective ricin A-chain immunotoxins. The screening was performed using an indirect assay in which the cells were treated firstly with the test antibody and then with a Fab' immunotoxin directed against mouse immunoglobulin. Only two antibodies, MEM75 and 120-2A3, both directed against the transferrin receptor (TfR) were predicted to form immunotoxins that would inhibit protein synthesis by the cells by 50% at a concentration (IC_{50}) of 10^{-8} M or less. This prediction was subsequently confirmed using several of the antibodies directly conjugated to ricin A-chain. By contrast, the same immunotoxins were highly toxic to non-myeloid cells which shared the target antigens. A comparison was made between the rates of endocytosis and degradation by HL60 cells of an anti-TfR immunotoxin 120-2A3·dgA, that was effective at killing myeloid cells, and a CD33 immunotoxin, p67-7·dgA, that bound to myeloid cells but did not kill them. The difference in potency of the two immunotoxins on HL60 cells was not due to deficient uptake of p67-7·dgA but was probably due to the more rapid intracellular degradation of p67-7·dgA. Fast and effective degradation in lysosomes, if a general finding, could explain the poor susceptibility of myeloid cells to ricin A-chain immunotoxins.

Key words: Acute myeloid leukaemia, ricin A-chain, immunotoxin, CD33, transferrin receptor, endocytosis.

INTRODUCTION

SECOND generation ricin A-chain immunotoxins which have better stability [1], higher purity and reduced liver homing [2] than their predecessors, have been demonstrated in mouse tumour models to be extremely effective in treating B-cell lymphomas [3, 4], T-cell lymphomas [5], and Hodgkin's disease [6] and are now undergoing clinical evaluation in man.

Despite their efficacy in lymphocytic leukaemia and lymphoma models, few attempts have been made to prepare immunotoxins for the treatment of myeloid tumours, such as acute myeloid leukaemia (AML) [7]. Specific targeting to AML cells has been hampered by the heterogeneity of their marker antigens and by the paucity of antibodies that recognize myeloid tumour-associated antigens which are absent from pluripotent stem cells. The most reliable markers to date appear to be CD13 and CD33 since these antigens seem to be expressed by early progenitors of myeloid blasts but not on very early pluripotent stem cells [8]. In the present study we screened two CD13 antibodies, three CD33 antibodies and 14 monoclonal antibodies against other myeloid tumour-associated antigens for their potential as ricin A-chain immunotoxins. Four myeloid cell lines were used as targets: the erythroleukaemia line, K562; the monocytic leukaemia line, U937, and the myeloid leukaemia lines, HL60 and KG1. The screening was performed by an indirect cytotoxicity assay which reliably predicts the potency of any given antibody as a ricin A-chain immunotoxin [9]. We demonstrate that, of the 19 antibodies tested, only the tw antibodies recognizing the transferrin receptor (TfR) made effective ricin A-chain immunotoxins. The failure of the ther antibodies t form effective immun toxins appears to be due to a generalized resistance f myeloid cells to ricin A-chain immunotoxins, since non-myeloid cells which shared

Abbreviations: IC_{50} concentration of which 50% of the protein synthesis is inhibited; *AML*, acute myeloid leukaemia; *TfR*, transferring receptor; *SMPT*, 5-succinimidyl-xycarbonyl-α-methyl-α-(2-pyridyldthio) toluene; *PBS*, phosphate-buffered saline; *SDS-PAGE*, sodium dodecyl sulfat polyacrylamide gel electrophoresis.

**Present address:* Medizinische Universitätsklinik I, 5000 Köln 41, F.R.G.

†*Present address:* Cancer Immunobiology Center, University of Texas Southwestern, 5328 Harry Hines Blvd, Dallas, TX 75295, U.S.A.

TABLE 1. MONOCLONAL ANTIBODIES EVALUATED IN THE PRESENT STUDY

	Antibody	Reactivity	Isotype	Source r reference
Myeloid antigens	My7	CD13	IgG2b	Coulter Imm.
	WM15	CD13	IgG1	Sera. Lab.
	1G10	CD15	IgM	Bernstein [10]
	T5A7	CDw17	IgM	Bernstein [11]
	My9	CD33	IgG2b	Coulter Imm.
	WM53	CD33	IgG1	Sera Lab
	p67-7	CD33	IgG1	Bernstein [11]
	12.8	CD34	IgM	Bernstein [11]
	E1	Uncl.	n.k.	Bernstein
	E2	Uncl.	n.k.	Bernstein
	E3	Uncl.	n.k.	Bernstein
Activation antigens	B-B10	CD25	IgG1	Wijdenes [13]
	HRS-3	CD30	IgG1	Pfreundschuh [14]
	MEM-75	CD71	IgG1	Horejsi [15]
	120-2A3	CD71	IgG1	Villela [15]
Various	RFB4	CD22	IgG1	Shen [3]
	5F1	CD36	IgM	Bernstein [10]
	IB3	Uncl.	IgG2a	Bernstein [12]
	TDR31·1	MHC class II	IgG1	Bodmer

Uncl. = unclustered.
n.k. = not known.

the target antigens were sensitive to the same immunotoxins. It is possible that myeloid cells tend to degrade immunotoxins rather than transport the A-chain to the cytosol.

MATERIALS AND METHODS

Materials

Blue Sepharose CL-6B, Sepharose G25 (fine grade), and Sephacryl S200 HR were obtained from Pharmacia Ltd (Milton Keynes, U.K.). An immunotoxin prepared by linking the Fab' fragment of affinity purified goat anti-m use immunoglobulin to deglycosylated ricin A-chain (dgA) was kindly provided by Dr Ellen Vitetta, University f Texas Southwestern Medical Center, Dallas, TX, U.S.A. The Fab' GAMIg·dgA was reactive with mouse IgM and IgG of all subclasses. Carrier-free N_r [^{125}I] and L[4, 5 ^{3}H] leucine (TRK 170) were purchased from Amersham International (Amersham, U.K.).

Monoclonal antibodies

Nineteen monoclonal antibodies of various CD clusters were used in this study. The specificity, class/subclass and source of the antibodies are listed in Table 1. Antibody E1 recognizes an unclustered antigen on myeloid cell lines and NALM-6 cells which is also present on stromal cells, endothelial cells and on 10–20% of human bone marrow cells. Antibody E2 is similar to E1 except that it also reacts with activated T cells. Antibody E3 recognizes an unclustered antigen present on certain myeloid cell lines and Daudi cells, and n stromal cells, endothelial cells, fibroblasts and 2–3% of human bone marrow cells (I. Bernstein, personal communicati n).

Cell lines

The cell lines used in this study were the erythroleukaemia cell line K562 [17], the monocyte cell line U937 [18], and the myeloid cell lines HL60 [19] and KG1 [20]. The cell lines were maintained in RPMI 1640 supplemented with 10% (K562, U937) or 20% (HL60, KG1) foetal calf serum (v/v), 4 mM L-glutamine, 200 units/ml penicillin, and 100 μg/ml streptomycin.

FACS analyses

Cells were suspended at 2×10^6/ml in PBS containing 0.2% (w/v) BSA and 0.2% (w/v) sodium azide (PBS/BSA/N_3). One hundred microlitres of cell suspensions were distributed into the wells of 96-well microtitre plates. One hundred microlitres of solutions of antibodies at a range of concentrations were added and the cells were incubated for 15 min at 4°C. The cells were washed three times with PBS/BSA/N_3^-, and were treated with FITC-labelled goat anti-mouse immunoglobulin for 15 min at 4°C. The cells were then washed three times and analysed on a FACS IV (Becton Dickinson, Oxnard, U.S.A.). The maximal fluorescence intensity that each of the 19 antibodies produced under saturating conditions was recorded.

Preparation of immunotoxins

Immunotoxins were prepared as described previously [21]. In brief, the antibodies were reacted with the heterobifunctional linker, SMPT, under conditions that introduced an average of 1.7 activated disulphide groups per molecule of antibody. The derivatized antibodies were then separated from unreacted material by gel chromatography on a Sephadex G25 column in PBS and mixed with excess freshly reduced, chemically deglycosylated ricin A-chain (dgA) in PBS. After 72 h at room temperature, th immunotoxin preparation was fractionated on a Sephacryl S200 HR column to remove unreacted A-chain and high m lecular weight aggregates. Free antibody was removed from the immunotoxin component by chromatography on Blue Sepharose CL-6B [22]. Analyses of the purified immunotoxin preparations by SDS–PAGE sh wed that the immunotoxins mainly (>90%) consisted of material having one molecule of antibody linked to one molecule f dgA.

Indirect cytotoxicity assay

The indirect cytotoxicity assays were performed as described by Till *et al.* [9]. Serial dilutions of unconjugated antibodies (2×10^{-8}–2×10^{-13} M) were prepared in cold RPMI 1640 medium, supplemented with 10 or 20% v/v foetal calf serum, 4 mM L-glutamine, 200 units/ml penicillin, and 100 µg/ml streptomycin ("complete medium"). The antibodies were distributed in 100 µl volumes in triplicate into 96-well plates and 100 µl of cell suspension containing 6×10^4 cells/ml in the same medium were added to each well. The plates were incubated at 4°C for 30 min. Fab'-GAMIg-A was then added to give a final concentration of 2 µg/mL. The plates were incubated for 24 h at 37°C in an atmosphere of 5% CO_2 in humidified air. Each culture was subsequently pulsed with 1 µCi [^{3}H]-leucine for a further 24 h. The cells were then harvested onto glass filters using a Titertek cell harvester and the radioactivity on the filters was measured using a liquid scintillation counter (1205 Betaplate LKB, Finland). The concentration (IC_{50}) at which the [^{3}H] leucine incorporation was inhibited by 50% relative to control cultures treated with Fab'-GAMIg-dgA alone was calculated.

Direct cytotoxicity assay

RFB4·dgA (CD22), B-B10·dgA (CD25), HRS3·dgA (CD30), p67-7·dgA (CD33), 120-2A3·dgA (CD71) and TDR31·1·dgA (MHC class II) immunotoxins were tested in direct cytotoxicity assays. In brief, immunotoxins were distributed in 100 µl aliquots in 96-well plates in concentrations ranging from 2×10^{-8} to 2×10^{-13} M in complete medium. Cell suspensions containing 6×10^4 cells in 100 µl of complete medium were added and the plates were incubated for 24 h at 37°C. The cultures were then pulsed with [^{3}H] leucine and harvested as described above. The concentration (IC_{50}) required to achieve a 50% reduction of protein synthesis relative to untreated control cultures was calculated.

Radioiodination

Monoclonal antibodies and immunotoxins were labelled with carrier-free Na [^{125}I] to a specific activity of approximately 1 µCi/µg using the Iodo-Gen reagent [23].

Endocytosis assay

Endocytosis studies using the HL60 cell line were performed as described by Press *et al.* [24]. The antibodies used were P67-7 (CD33), 120-2A3 (CD71), and IRac (non-binding control) and their corresponding ricin A-chain immunotoxins. Cells were washed in cold serum-free RPMI 1640 medium and incubated with [^{125}I]-labelled antibodies or immunotoxins (50 ng/10^6 cells) at 4°C for 30 min. After two washes, the cells were suspended at 2×10^6 cells/ml in complete medium and were transferred in 0.5 ml volumes to sterile 2 ml tubes (Sarstedt). The tubes were then incubated at 37°C in an atmosphere of 5% CO_2 in humidified air for various time intervals.

After 0, 0.5, 1, 2, 4, 8 and 24 h of incubation, the cells were centrifuged and 200 µl of supernatant was removed for TCA precipitation (see below). The cells were washed twice in ice-cold serum-free medium and cell surface-bound antibody or immunotoxin was then removed by two consecutive washes with acid-papain buffer (0.05 M glycine–HCl, 0.1 M NaCl, 2.5 mg/ml papain, pH 2.5). The two acid washes were pooled and th radioactivity of the acid washes and that associated with the cell pellet was measured using a Packard Multi-Prias gamma counter. These procedures released >90% of the cell-associated radioactivity when cells were kept at 4°C to inhibit endocytosis. Non-specific binding of labelled IRac antibody amounted to only 1% of the radioactivity of 120-2A3 r P67-6 bound to HL60 cells.

The extent of degradation of the [^{125}I]-labelled antibodies and immunotoxins was assessed by treating 200 µl of culture supernatants with 500 µl of 25% (v/v) TCA for 30 min at 4°C. The mixture was centrifuged and the pellet was washed with a further 0.5 ml of 25% TCA at 4°C. The radioactivity of the pellet and the two TCA washes from each sample were counted separately.

TABLE 2. FACS ANALYSES ON MYELOID CELL LINES

Antibody	Specificity	Intensity of staining			
		K562	U937	HL60	KG1
My7	CD13	–	++	++	++
WM15	CD13	–	++	+++	++
1G10	CD15	+	++	++	++
T5A7	CD17	+	–	++	++
RFB4	CD22	–	–	+	–
B-B10	CD25	–	++	–	–
HRS-3	CD30	++	+	+	–
My9	CD33	+	++	++	++
WM53	CD33	+	++	+++	+++
P67-7	CD33	+	+++	+++	+++
12.8	CD34	–	+	–	++
5F1	CD36	+	+	+	+
1B3	Uncl.	–	+	+	+
E1	Uncl.	–	+	+++	+++
E2	Uncl.	–	++	+	++
E3	Uncl.	–	+	+	+
TDR31·1	MHC Class II	+	+	–	++
MEM75	CD71	+++	+++	+++	+
120-2A3	CD71	+++	+++	+++	++

Symbols: (–) = MFI <20U; (+) = MFI 21–70 U; (++) = MFI 71–120 U; (+++) = MFI >120 U.
Uncl. = unclustered.

RESULTS

Reactivity of antibody panel with myeloid leukaemia

Table 2 lists the intensities of staining of K562, U937, HL60, and KG1 cells by the 19 monoclonal antibodies in the panel.

Of the antibodies that recognized myeloid cell-associated antigens, the CD33 antibodies, p67-7, WM53 and My9, gave the strongest and most c nsistent staining of all four cell lines. The CD13 MoAbs, My7 and WM15, bound moderately to strongly to U937, HL60, and KG1, but did not bind to the K562 cell line. The CD15 antibody, 1G10, stained U937, HL60 and KG1 cells moderately and K562 cells weakly. The CD17 antibody, T5A7, showed moderate staining of HL60 and KG1 cells and weak staining of K562 cells. Th CD34 m noclonal antibody, 12.8, gave moderate staining f KG1 cells

and weak staining of U937. Four hitherto unclustered antibodies against myeloid cell-associated antigens, 1B3, E1, E2, and E3, gave mixed staining patterns on U937, HL60, and KG1 cells, but did not stain K562.

Of the antibodies against leukocyte activation antigens, those against the transferrin receptor (CD71), 120-A3 and MEM75, gave strongest staining of K562, U937 and HL60. The CD30 antibody, HRS-3, showed moderate staining of K562, weak staining of HL60 and U937, and no staining of KG1. The CD25 antibody, B-B10, only stained U937.

The antibody against a monomorphic determinant n MHC class II, TDR31·1, weakly stained K562 and U937 and gave moderate staining of HL60. Finally, the CD22 antibody, RFB4, weakly stained HL60 cells.

Lack of potency of ricin A-chain immunotoxins for myeloid cells as predicted by the indirect cytotoxicity assay

In this indirect method for screening antibodies f r their potential to form effective ricin A-chain immunotoxins, target cells are incubated first with the test antibody and then with the Fab'-GAMIg-dgA immunotoxin. The immunotoxin binds to the test antibody and enters the cells by the same or a similar route as would a primary immunotoxin constructed from the test antibody. Thus, the assay accurately predicts the potency of direct ricin A-chain immunotoxins [9]. As shown in Table 3, only the indirect immunotoxin prepared from the anti-transferrin receptor antibodies, 120-2A3 and MEM-75, were toxic to the four cell lines tested. Their t xicity towards U937 and HL60 cells was weak, with IC_{50} values of between 10^{-8} and 10^{-9} M, whereas their toxicity towards K562 and KG1 cells was moderate, with IC_{50} values generally between 10^{-9} and 10^{-10} M. Indirect immunotoxins prepared from all the other antibodies in the panel were completely ineffective against the U937, HL60 and KG1 cells. Similarly, they were mostly ineffective against K562 cells with only 1G10, T5A7 and 5F1 giving weak t xicity.

Lack of potency of directly linked ricin A-chain immunotoxins on myeloid leukaemia cell lines

Table 4 lists the IC_{50} values of the directly linked ricin A-chain immunotoxins on myeloid leukaemia cell lines and non-myel id cell lines. All of the immunotoxins retained m re than 50% of the binding activity of the native antibodies, as judged by FACS analyses (see Methods). As predicted by the indirect assay, the anti-TfR immunot xin, 120-2A3·dgA,

TABLE 3. POTENCY OF RICIN-A-CHAIN IMMUNOTOXINS AS PREDICTED BY AN INDIRECT CYTOTOXICITY ASSAY ON MYELOID CELL LINES

Antibody	Specificity	Predicted Cyt t xic Potency			
		K562	U937	HL60	KG1
BF10	CD13	–	–	–	–
My7	CD13	–	–	–	–
WM15	CD13	–	–	–	–
1G10	CD15	+	–	–	–
T5A7	CD17	+	–	–	–
RFB4	CD22	–	–	–	–
B-B10	CD25	–	–	–	–
HRS-3	CD30	–	–	–	–
My9	CD33	–	–	–	–
WM53	CD33	–	–	–	–
p 67-7	CD33	–	–	–	–
12.8	CD34	–	–	–	–
5F1	CD36	+	–	–	–
1B3	Uncl.	–	–	–	–
E1	Uncl.	–	–	–	–
E2	Uncl.	–	–	–	–
E3	Uncl.	–	–	–	–
TDR31·1	MHC Class II	–	–	–	–
MEM-75	CD71	++	+	+	+
120-2A3	CD71	++	+	+	++

Symbols: (–) = IC_{50}: $>10^{-8}$ M; (+) = IC_{50}: 10^{-8}–10^{-9} M; (++) = IC_{50}: 10^{-9}–10^{10} M.

was toxic to the cells. It inhibited their protein synthesis by 50% at concentrations of 5×10^{-11} M for K562, 6×10^{-11} M for KG1, 2×10^{-10} for HL60, and 4×10^{-9} M for U937. By contrast, and again as predicted by the indirect assay, the CD33 immunotoxin, p67·7-dgA, was practically devoid of toxicity to any of the cell lines despite the fact that the P67·7-dgA antibody bound more strongly to the cells than did any of the other antibodies tested. In addition, the other directly linked ricin A-chain immunotoxins, RFB4·dgA (CD22), B-B10·dgA (CD25), HRS-3·dgA (CD30), and TDR31·1·dgA (MHC class II), which bound weakly to moderate to some of the myeloid cell lines (Table 2), were all devoid of cytotoxic effects. All four cell lines were sensitive to ricin itself, which gave IC_{50} values of 2×10^{-11}–5×10^{-13} M (data not shown), indicating that the cells had sensitive ribosomes and possessed machinery to transport the A-chain to the cytosol. Thus it appears that the resistance of the myeloid cells is because they do not permit the immunotoxins to enter a cellular compartment from which the A-chain can enter the cytosol.

Sensitivity of non-myeloid cells to killing by ricin A-chain immunotoxins directed against myeloid antigens

In sharp contrast to their lack of t xicity to myel id cells, RFB4·dgA, HRS-3·dgA, B-B10·dgA and

TABLE 4. CYTOTOXIC EFFECTS OF DIRECTLY LINKED IMMUNOTOXINS ON MYELOID AND NON-MYELOID CELL LINES

Immun t xin	Cell line	Type*	Staining intensity (MFI)	IC_{50} (M)
120-2A3·dgA	K562	M	+++	5×10^{-11}
	U937	M	+++	4×10^{-9}
	HL60	M	+++	2×10^{-10}
	KG1	M	++	6×10^{-11}
	L540	HD	++	2×10^{-10}
p67-7·dgA	K562	M	+	1×10^{-8}
	U937	M	+++	2×10^{-8}
	HL60	M	+++	2×10^{-8}
	KG1	M	+++	2×10^{-8}
	L540	HD	−	$>10^{-7}$
RFB4·dgA	HL60	M	+	$>10^{-7}$
	ARH-77	L	+	2×10^{-11}
	NALM-6	L	+	2×10^{-11}
	Daudi	L	+++	1×10^{-12}
HRS-3·dgA	K562	M	++	$>10^{-7}$
	U937	M	+	$>10^{-7}$
	HL60	M	+	$>10^{-7}$
	L540	HD	+++	8×10^{-11}
B-B10·dgA	U937	M	++	$>10^{-7}$
	L540	HD	+++	4×10^{-11}
TDR31·1·dgA	K562	M	+	$>10^{-7}$
	U937	M	+	$>10^{-7}$
	KG1	M	++	$>10^{-7}$
	L540	HD	++	2×10^{-10}
OX7·dgA	K562	M	−	$>10^{-7}$
	U937	M	−	$>10^{-7}$
	HL60	M	−	$>10^{-7}$
	KG1	M	−	$>10^{-7}$

* Symbols: M = myeloid cell line; HD = Hodgkin's disease cell line; L = lymphoid cell line.

TDR 31·1·dgA were highly toxic to non-myeloid cells which expressed the target antigens at similar levels to those on myeloid cells (Table 4). The IC_{50} values on the non-myeloid cells ranged from 2×10^{-10} to 2×10^{-12} M, indicating that the non-myeloid cells were typically more than 2000-fold more sensitive to the immunotoxins than were the myeloid cells.

Kinetics of uptake and metabolism of CD33 and CD71 antibodies and immunotoxins by HL60 cells

Possible explanations for the generalized low potency of immunotoxins on myeloid cells could be that these cells fail to endocytose the immunotoxin, r do so but to a compartment (e.g. the lysosomes) unfavourable for A-chain transport to the cytosol. We therefore compared the kinetics of uptake and metabolism of the 120-2A3 antibody and immunotoxin (strong binding, moderate potency) with that f the P67-7 antibody and immunotoxin (strong binding, low potency) and the HL60 cell line. These kinetic data are illustrated in Fig. 1.

Both the p67-7 antibody and the p67-7 ricin A-chain immunotoxin were rapidly internalized by HL60 cells and degraded. Only 20% of the [^{125}I]-labelled antibody and the immunotoxin were present on the cell surface after 4 h at 37°C. The antibody and the immunotoxin accumulated within the cells progressively over the first 2 h, and then the label declined. The decline was mirrored by the appearance of TCA soluble radioactivity in the supernatant (Fig. 1a, c), indicating that the antibody and immunotoxin which had been internalized were being degraded presumably through the action of lysosomal enzymes. The amount of acid-soluble material in the supernatant reached 56% of the total applied radioactivity for the p67-7 antibody and 65% for the p67-7·dgA immunotoxin after 24 h of incubation (Fig. 1a, c).

The anti-transferrin receptor antibody, 120-2A3, and the 120-A3·dgA immunotoxin were int rnalized and degraded less rapidly than the p67-7 antibody and immun toxin. After 4 h, 33.2% f the antibody and 28.4% f the immunotoxin were present on the

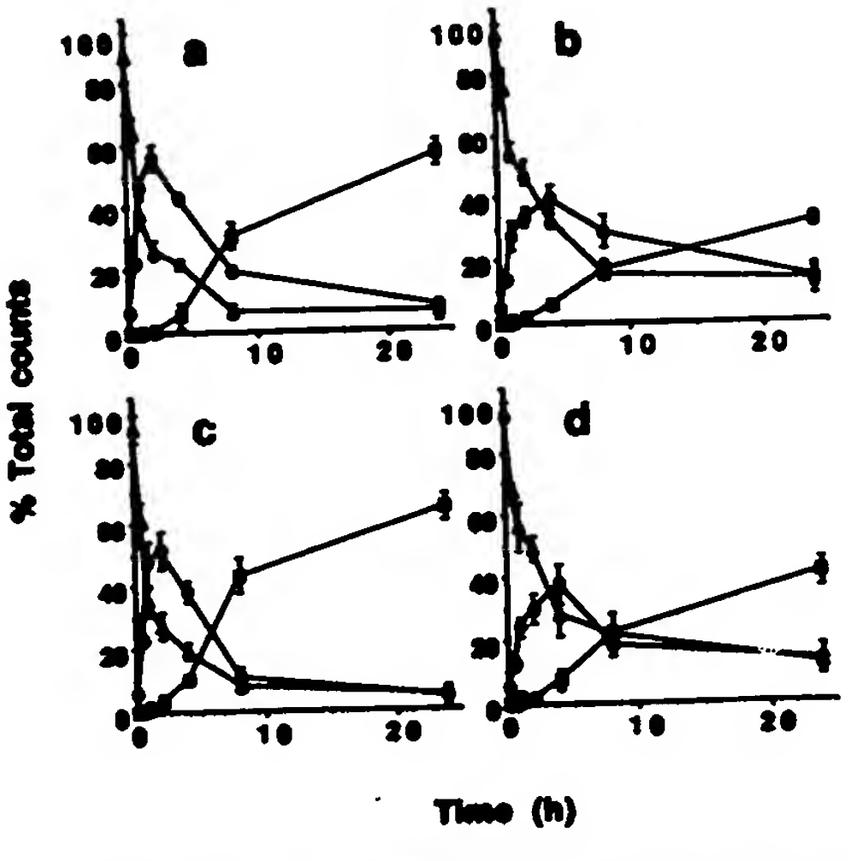

FIG. 1. Endocytosis and catabolism of CD33 antibody, p67-7 (a), and anti-TfR antibody, 120-2A3 (b), and their corresponding immunotoxins prepared from deglycosylated ricin A-chain, p67-7·dgA (c), and 120-2A3·dg17 (d) by HL60 cells. The cells were coated with ^{125}I-labelled antibodies or immunotoxins at 4°C and were then washed and incubated at 37°C for various time intervals. The percentage of the total radioactivity which was present on the cell surface (▲), inside the cell (●) or which was present in degraded (TCA-soluble) form in the tissue culture medium (■) was determined as described in Methods. Points, mean values of three experiments; bars, SD.

cell surface, compared with 21.8% and 19.3% for the 67-7 antibody and immunotoxin respectively. The amount of TCA-soluble radioactivity that was present in the supernatant was 32% for the 120-2A3 antibody and 41% for the immunotoxin compared with 56% and 64% for the p67-7 antibody and immunotoxin respectively.

Thus, the lack of cytotoxicity of the CD33 immunotoxin, p67-7·dgA, compared with the CD71 immunotoxin, 120-2A3·dgA, appears to be due, at least in part, to the more efficient intracellular degradation of the CD33 immunotoxin.

DISCUSSION

The major finding to emerge from this study was that human myeloid leukaemia cell lines are generally not susceptible to killing by ricin A-chain immun toxins. From a panel of 19 monoclonal antibodies nly two, both recognizing the transferrin receptor (TfR), showed significant toxicity to four myeloid leukaemia cell lines.

A comparison of the rates f endocytosis of an ineffective CD33 immunot xin, p67-7·dgA, with an effective CD71 immun t xin, p120-2A3·dgA, directed against the TfR, revealed that the p67-7 immun toxin was endocytosed m re rapidly by HL60 cells than the 120-2A3 immunot xin. Thus the failure f p67-7·dgA to kill HL60 and other myel id cells was n t due to a deficiency in endocytosis, as has been reported t be the explanation for the lack of efficacy of A-chain immunotoxins in certain other systems [25]. However, after endocytosis, the p67-7·dgA was degraded to TCA-soluble material about twice as rapidly as 120-2A3·dgA. This suggests that the lack of activity of p67-7·dgA is because it is routed to the lysosomes and catabolized rather than being routed to an intracellular compartment (possibly the trans-Golgi region [26]) from which the A-chain can be transported to the cytosol.

The slower uptake and catabolism rate of the anti-TfR immunotoxin, 120–2A3·dg17, accords with the finding by Raso *et al.* [27] with CEM cells, that anti-TfR-A-chain immunotoxins, like transferrin itself, recycle back to the cell surface and are only slowly degraded. Others have shown that transferrin itself coupled to ricin A-chain [28] or Pseudomonas exotosin A [29] is highly toxic to various cell types. Thus the sensitivity of myeloid cells to the anti-TfR immunotoxins may be because they, like other cell types, have a nutritional requirement for transferrin-Fe^{3+} and employ uptake mechanisms that enable the ligands to evade lysosomal capture.

Non-myeloid cells also vary in their sensitivity to ricin A-chain immunotoxins depending on the affinity of the immunotoxin for the target antigen [3, 6], the density of the target antigen on the cell surface [30], the location of the epitope recognized by the immunotoxin on the target antigen [24], and the rate and route of endocytosis of the antigen–immunotoxin complex by the cell [31]. Consequently, some ricin A-chain immunotoxins are potently toxic to non-myeloid cells, whereas others are not. However, the resistance of myeloid cells to ricin A-chain immunotoxins appears to be much more generalized. This difference is emphasized by our finding that CD22, CD25, CD30, and anti-MHC class II ricin A immunotoxins, which are highly toxic to normal and malignant B cells and T cells and to Hodgkin lymphoma cells [3–6], had no cytotoxic effects against myeloid cell lines expressing these antigens. It is possible that the generalized resistance of myeloid cells is related to their physiological role, which is to scavenge and destroy. Myeloid cells have a particularly well-developed lysosomal machinery and perhaps the majority of molecules which bind to these cells (with the notable exception of transferrin-related compounds) are destined for lysosomal destruction.

Alth ugh anti-TfR immunotoxins were moderately toxic to myeloid cells, they can pr bably not be used for systemic therapy in malignant diseases

because the TfR is expressed on vital tissues such as brain endothelial cells, ndocrine pancreas, Kupffer cells and hepatocytes [15]. Thus the therapeutic use f anti-TfR immun toxins is probably confined to intra-regional therapy, as in the treatment of peritoneal ascitic tumours [32], or to purging bone marrow of dividing cells [7].

Future steps in the development of immunotoxins for the treatment of myeloid leukaemia should include screening the immunotoxins on fresh AML cells, since cell lines could differ from fresh blasts in their sensitivity. Indeed, Demur *et al.* [33] have reported that fresh AML blasts have greater sensitivity than K562 or KG1 cells to a ricin A-chain immunotoxin directed against MHC class II. A further possibility would be to harness the ability of ricin B-chain to potentiate A-chain entry. As we [34, 35] and others [32, 36] have shown previously, wh le ricin immunotoxins with blocked galactose binding sites can be powerfully and specifically cytotoxic in situations where the analogous A-chain immunotoxins are ineffective. Such an approach could be especially suitable for myeloid cells because there is evidence that blocked ricin immunotoxins may act by being degraded proteolytically inside the cell to release active ricin (which is resistant to proteolysis) which then kills the cell [34].

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Antileukemic activity of recombinant humanized M195-gelonin immunotoxin in nude mice

Y Xu[1], Q Xu[1], MG Rosenblum[2] and DA Scheinberg[1]

[1]Memorial Sloan-Kettering Cancer Center, NY; and [2]Immunopharmacology Section, Department of Clinical Immunology and Biological Therapy, MD Anderson Cancer Center, Houston, TX USA

A leukemia-selective immunotoxin was constructed by linking recombinant gelonin (rGel), a single chain ribosome inhibitory protein, to recombinant humanized M195 antibody (HuM195), which recognizes the cell-surface protein designated CD33. CD33 is an antigen found on myeloid leukemia blasts as well as myeloid progenitor cells but it is not expressed in detectable amounts on the ultimate hematopoietic progenitor stem cell. Our previous studies indicated that a non-recombinant humanized immunotoxin displayed specific, potent toxicity towards CD33-positive cells but not to CD33-negative cells *in vitro*. In the current study, a recombinant humanized immunotoxin, HuM195-rGel, was evaluated *in vivo* in a nude mouse model of human myeloid leukemias. HuM195-rGel was found to target leukemia cells rapidly *in vivo* and was subsequently internalized into the cells. For trials *in vivo*, nude mice were injected (ip) with 10^7 log-phase HL60 human leukemia cells 10 days prior to the start of i.p. HuM195-rGel treatments. HuM195-rGel demonstrated significant tumor suppressive activity in this model. While all mice treated with either saline, rGel alone, or HuM195 plus unconjugated rGel (at 10 or 14 days after transplantation) had rapid tumor growth or early deaths, 50% of mice treated with HuM195-rGel failed to develop leukemic tumors for 5 months and the other 50% had significantly retarded tumor growth after treatment with HuM195-rGel. Mice treated at later times (28 days after transplantation of leukemia cells) also showed delayed leukemia cell growth, but no cures. These data show that HuM195-rGel can target leukemia cells *in vivo* and can result in pronounced anti-leukemic effects.

Keywords: myeloid leukemia; CD33; immunotoxin; gelonin; HuM195

Introduction

Immunotoxins (IT) are a class of proteins that consist of a monoclonal antibody (mAb) covalently linked or genetically fused to a cytotoxic molecule and are thus able to direct potent cytotoxic protein to particular cells.[1,2] Ribosome inhibitory proteins (RIPs) can be specifically targeted to certain tissues through chemical conjugation or genetic fusion with mAb and thereby acquire cell-specific cytotoxicity.[3,4]

Gelonin toxin originally isolated from the seeds of *Gelonium multiflorum* is a single polypeptide chain in a class of proteins designated type I RIPs. Unlike the type II RIPs, for example ricin, which is composed of a ricin A chain and a lectin-like B chain, gelonin has a relatively low native cytotoxic activity due to the lack of a lectin B chain, which can nonspecifically bind to cell membrane glycoproteins. Like all plant-derived RIPs, gelonin damages 28S rRNA through a glycosidase that cleaves the glycosidic bond at a unique adenine base in the rRNA, thereby inhibiting protein synthesis.[5,6] Recently, the native gelonin protein was sequenced, a synthetic gene encoding gelonin was sythesized, and biologically active recombinant gelonin (rGel) was synthesized in *E. coli*.[7]

Gelonin has several advantages for use in immunotoxin therapy compared to other RIPs, including the lack of the B chain containing the galactose-specific lectin domain responsible for much non-specific binding and toxicity.[4,5] As a result, free gelonin is much less toxic to intact mammalian cells *in vitro* and *in vivo* than type II RIP. Despite this relative safety, in a cell-free rabbit reticulocyte translation assay, gelonin demonstrates nearly equal biological activity to heterodimeric toxins.[8,9] In addition, immunotoxins containing an A chain separated from a B chain are, in general, less potent on intact cells[10,11] and the larger heterodimers may also be more immunogenic than the single chain toxins. Therefore, gelonin may have properties advantageous for the generation of potent and specific immunoconjugates.[12]

CD33 is a useful target antigen for therapy of myelogenous leukemias, as it is expressed on the cell surface of greater than 80% of leukemia isolates from patients with myeloid leukemia with an average antigen density of 10 000 sites per cell.[13–16] However, CD33 is not found on tissues outside the hematopoietic system.[13] Its expression within the hematopoietic system is limited to early myeloid progenitor cells, monocytes and dendritic cells. Importantly, CD33 is not found on the ultimate hematopoietic progenitor stem cell, thus allowing in principle, selective elimination of leukemia cells and early progenitors while preserving capacity for long-term regeneration of marrow cells.[14,17,18]

HuM195, a humanized version of M195 constructed by genetically grafting the murine complementarity-determining region (CDR) to a human IgG1 framework and constant regions, is reactive with CD33.[19] The humanized antibodies may be advantageous due to reduced immunogenicity, higher avidity, and longer serum half lives.[19–22] In addition, rapid internalization occurs upon binding of mAb HuM195 to CD33 both *in vitro* and *in vivo*.[19,22,23] This suggests that HuM195 can be a suitable candidate for immunotoxin studies in humans.

We recently described a HuM195-gelonin immunotoxin.[12] HuM195-gelonin did not completely prevent hematopoietic reconstitution *in vitro* after treatment as evidenced by bone marrow colony experiments, but bone marrow treated with HuM195-gelonin demonstrated a log reduction of colony formation. This is expected since colony forming unit-granulocyte monocytes (CFU-GM) express CD33.[13,15,24]

Clinical trials using ^{131}I labeled M195 have demonstrated rapid and specific localization of antibody to tumor sites, saturation of all available CD33 antigen, followed by intracellular internalization.[22,23] Dose escalation studies using ^{131}I-M195 resulted in greater than 99% killing of leukemic blasts with negligible toxicity outside of the hematopoietic compartments.[25] However, due to the long range cytotoxicity of the conjugated nuclide (approximately 50 cell diameters) killing of normal bystander cells occurs as well, requiring bone mar-

Correspondence: DA Scheinberg, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10021, USA
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row transplantation at high doses. We propose that use of an anti-CD33 immunotoxin may avoid this problem by targeting cells bearing the CD33 antigen. In this paper, we describe the activity of immunotoxins constructed by chemical conjugation of HuM195 with recombinant gelonin in a mouse model of HL60 leukemia.

Materials and methods

Animals

Six-week-old female outbred Swiss nu/nu mice were obtained from the colony at Sloan Kettering Institute. All bedding material was sterilized before use; the cages were covered with an air filter and maintained in isolation cabinets. Animal handling and experiments were performed in aseptic atmosphere using a laminar flow hood.

Cell lines

HL60 (acute myeloid leukemia, CD33 positive), RAJI and DAUDI (both B lineage Burkitt's lymphomas, CD33 negative) were maintained in culture using RPMI 1640 supplemented with 10% Serum Plus (JRH Biosciences, Lenexa, KS, USA) and 10% heat inactivated fetal calf serum (Intergen, Purchase, NY, USA).

Antibodies and radiolabeled antibodies

HuM195, a humanized IgG1 reactive with human CD33 antigen, and HuFd79, a genetically engineered human IgG1 construct reactive with a herpes simplex virus antigen not found on HL60 cells, were prepared as described.[19] Antibodies were trace labeled with ^{125}I (New England Nuclear, Boston, MA, USA) using the chloramine T method to a specific activity of 2–10 μCi/μg as described previously.[16] Immunoreactivity of the radiolabeled antibody was determined by incubating serial dilutions (10^7 to 10^6 cells) of HL60 cells with 2–4 ng of radiolabeled antibody for 1 h at 4°C. Cells were resuspended in phosphate buffered saline twice and the pellets were counted to determine total cell-bound ^{125}I-antibody.

Toxins

Recombinant gelonin (rGel) was derived and purified as described.[7] Functional activity studies demonstrated that this protein behaved similarly to chemically purified natural gelonin.[7]

Preparation of HuM195 Conjugates

HuM195 was conjugated with rGel using N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP); the immunoconjugates were purified by gel-permeation chromatography and separation on cibachron blue sepharose as previously described.[12] The molecular weight of HuM195-rGel was about 180 kDa demonstrating a 1 : 1 molar ratio of HuM195 to rGel.

Flow cytometry assays

Cells were washed and resuspended in 2% rabbit serum (Pel Freeze, Rogers, AK, USA) to reduce nonspecific binding. 5×10^5 cells in a final volume of 0.1 ml were incubated 1 on ice in the presence of primary antibody HuFd79 control or HuM195. Cells were washed twice, incubated 30 min o ice with secondary fluorescein isothiocyanate (FITC) labele antibody (goat anti-human immunoglobulin) (Kirkegaard an Perry, Gaithersburg, MD, USA), washed twice, and fixed wi 0.5% paraformaldehyde. FITC fluorescence intensity wa measured on an EPICS Profile II flow cytometer (Coulter Miami, FL, USA).[15]

Inhibition of tritiated thymidine or leucine incorporation

An aliquot containing 100 μl of cells were washed and incubated at 37°C in 96-well plates in the presence of 50 μl of antibody, conjugate or toxin. After an incubation time of 3–7 days, 50 μl of 10 μCi/ml of tritiated thymidine or leucine (Du Pont-New England Nuclear) was added to each well and allowed to incorporate for 5 to 6 h. Trichloroacetic acid was added to a final concentration of 10% to precipitate protein for ^{3}H-leucine incorporation experiments. Cells were harvested using a semiautomatic harvester (Skatron) and read in a scintillation counter LS 6000IC (Beckman, Fullerton, CA, USA).

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide : thiazolyl blue (MTT) assays

One hundred microliters of cells were washed and incubated at 37°C in 96-well plates in the presence of 50 μl of antibody, conjugate or toxin. After an incubation time of 3–7 days, the plate was centrifuged 5 min at 1000 r.p.m. MTT diluted in phosphate-buffered saline, was added to each well and incubated for 4 h. Plates were washed and the formazan product was solubilized with 0.04 M HCl in 2-propanol and quantitated spectrophotometrically at 570 nm.

Transplantation HL60 cells into nude mice

A 0.2-ml aliquot containing 10^7 HL60 cells from suspension culture was transplanted ip into nude mice. Tumors grew subcutaneously and the cutaneous tumor size was measured as a cross product to derive surface area. For studies *ex vivo*, pieces of tumor were minced and intact single cells were isolated on a Ficoll–Hypaque density gradient or after passage through a 70 μm nylon filter (Spectrum, Houston, TX, USA).

In vivo measurement of antibody targeting to tumor

The rate at which ^{125}I-HuM195 was bound to and subsequently internalized into leukemia cells *in vivo* was measured. Tumor bearing mice at 4 weeks (two mice per group) infused ip with 2 or 20 μg of ^{125}I-HuM195 or ^{125}I-HuFd79 (a negative control), were sacrificed at 4 or 24 h after the infusion. Tumors were excised and weighed at 4°C to avoid internalization during the assay process. Cell surface bound ^{125}I-HuM195 or ^{125}I-HuFd79 was then stripped using 50 mM

glycine/HCl, 150 mM NaCl, pH 2.8, and internalized c.p.m. (residual c.p.m. in the cell pellets) were calculated.[19] Specific surface bound and internalized HuM195 were calculated by subtraction of nonspecific surface bound and internalized HuFd79.

Immunotherapy

The test animals were treated i.p. with HuM195-rGel (36 μg/dose), recombinant gelonin (6 μg/dose), or HuM195 and recombinant gelonin mixture (30 μg HuM195 plus 6 μg gelonin/dose) twice a week in a final volume of 0.2 ml (the molar amounts of toxin and antibody were kept constant). Assuming a circulation volume of about 2 ml, the dose injected was equivalent to 100 nM initial concentration. Control mice were treated with 0.2 ml saline twice a week.

Results

In vitro cytotoxicity

HuM195-rGel was tested for its ability to kill CD33 positive and CD33 negative cells in comparison to free rGel. Activity and cytotoxicity were determined by inhibition of incorporation of ^{3}H-leucine into protein and by trypan blue exclusion. Dose–response curves were generated by testing the inhibitory effects HuM195-rGel on the protein synthesis of HL60 cells (CD33 positive) and RAJI (CD33 negative) in culture (Figure 1a). In the *in vitro* assays, the concentration of HuM195-rGel required to inhibit protein synthesis in HL60 cells by 50% was 0.6 nM, whereas the concentration of rGel alone required to nonspecifically inhibit protein synthesis in both HL60 and RAJI cells by 50% was about 200 nM (Figure 1a). In the concentration range of 10–100 nM HuM195-rGel, protein synthesis in HL60 cells was almost completely inhibited while no cytotoxicity was observed with the CD33 negative cell lines RAJI (Figure 1a) and DAUDI (not shown). However, HuM195 alone did not affect the protein synthesis in CD33 positive HL60 cells (Figure 1a). This shows that the inhibition of protein synthesis was due to specific binding and activity of the immunotoxin, and not a nonspecific property of the antibody itself. The specific targeting of leukemic cells by HuM195-Gel appeared to occur via the CD33 antigen binding site and not through the Fc region or other non-specific binding sites on target cells as shown previously.[12]

The cytotoxicity of HuM195-rGel was directly determined by trypan blue analysis. The concentration of HuM195-rGel required to kill 50% of cells was 0.7 nM (Figure 1b), similar to the concentration of HuM195 required to inhibit protein synthesis by 50% (Figure 1a). However, HuM195-rGel did not kill CD33 negative RAJI cells at the highest concentration of 100 nM, suggesting that it may be used safely for study *in vivo*. The cytotoxicity was also confirmed by ^{3}H-thymidine incorporation and MTT assays (not shown), confirming that HuM195-rGel causes HL60 leukemia cell death *in vitro*.

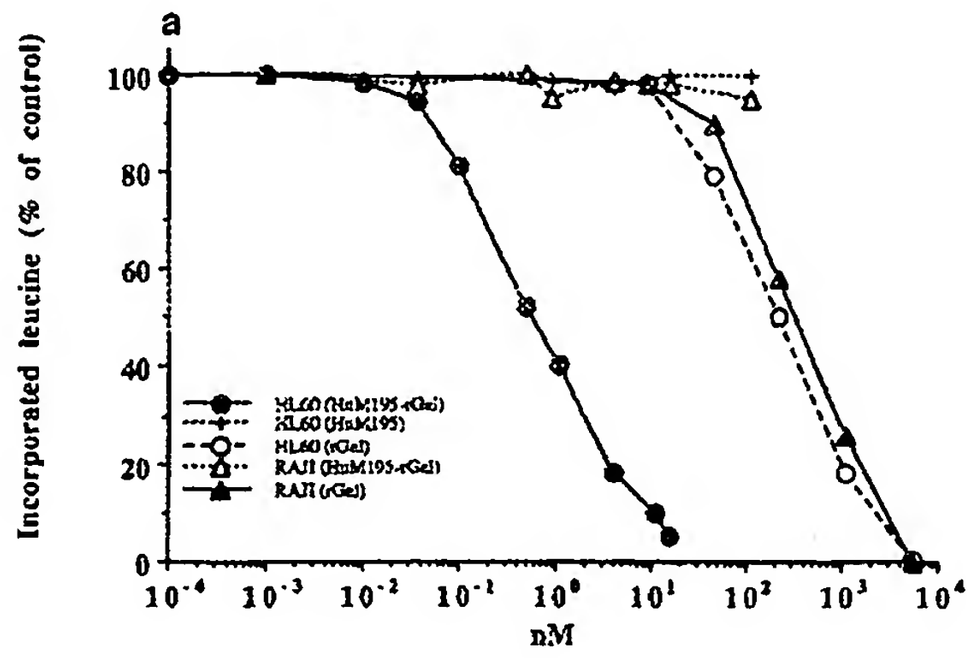

Figure 1 Cytotoxicity and inhibition of protein synthesis in HL60 or RAJI cells by recombinant gelonin (rGel), HuM195 and HuM195-rGel. (a) Inhibition of protein synthesis in HL60 or RAJI cells by rGel, HuM195 and HuM195-rGel. HL60 or RAJI cells at a final concentration of 10^5 cells/ml were incubated 3 days at 37°C in the presence of HuM195-rGel, HuM195 and rGel. Levels of protein synthesis were determined by 5-h incorporation of tritiated leucine into trichloroacetic acid precipitable protein. The treatment is shown in parenthesis. (b) Cell viability determined by trypan blue exclusion. HL60 or RAJI cells at a final concentration of 10^5 cells/ml were incubated 3 days at 37°C in the presence of HuM195-rGel. Trypan blue was added and live and dead cells were counted under the microscope.

Targeting of radiolabeled HuM195 into leukemic cells in vivo

We have previously shown that nude mice retain limited ability to generate antibodies to the HL60 cells after transplant and that CD33 can be down-regulated by this response.[26] Therefore, the expression of CD33 on the tumors was assessed. The cells from the leukemic tumors retained expression of CD33 positive antigen after growth *in vivo*, as determined by flow cytometry at saturating mAb concentrations (Figure 2). The internalization of ^{125}I-HuM195 into the target cells *in vivo* was rapid, and similar to the observations *in vitro*.[19] At 4 h after infusion of 2 or 20 μg antibody, 23–26% of bound ^{125}I-HuM195 was internalized, whereas a higher rate of internalization (38–43%) was seen at 24 h (Figure 3).

In vivo antitumor effects of HuM195-rGel

The leukemic cell growth in the subcutaneous space and peritoneum of nude mice was substantially reduced by HuM195-rGel. At 10 days after transplantation of HL60 cells into the peritoneum of nude mice, tumors of about 2 mm^2 in size are present in the subcutaneous space (Figure 4). After three injections of HuM195-rGel at a dose of 36 μg per mouse beginning at 10 days, two out of four mice did not develop tumors for

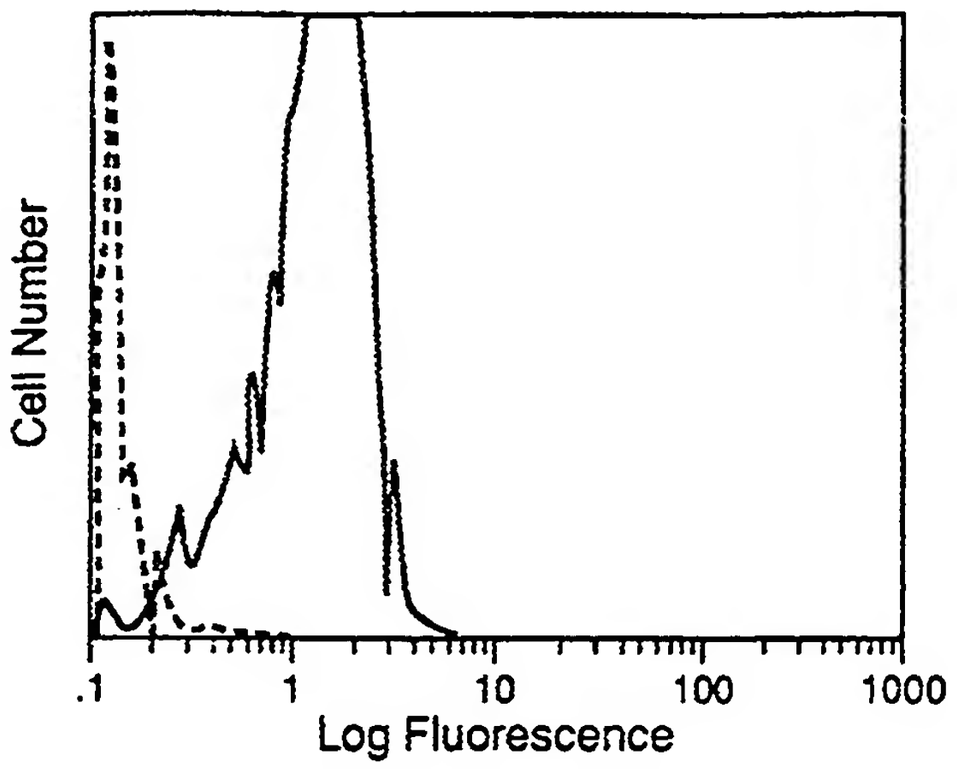

Figure 2 Tumor cell surface antigen expression. Expression of CD33 by HL60 cells grown at 4 weeks in a representative tumor mass *in vivo* determined by indirect immunofluorescence as described under Materials and methods:---, HuFd79;—, HuM195.

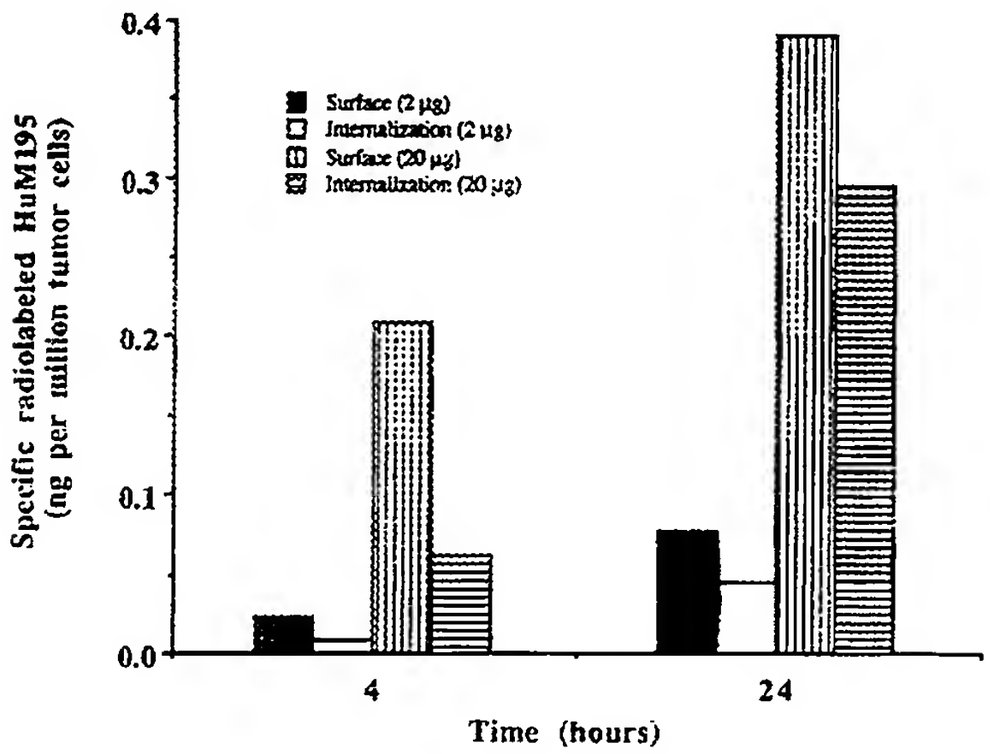

Figure 3 Specific binding and internalization of ^{125}I-HuM195 in tumor *in vivo*. Tumor bearing mice at 4 weeks after transplantation received infusions of 2 or 20 μg of ^{125}I-HuM195 shown in parenthesis. Mice were sacrificed at 4 or 24 h after the infusion. Tumors were excised, weighed, and counted. Specific surface-bound and internalized HuM195 were calculated as described under Materials and methods. S.D. was less than 10%.

up to 5 months after transplantation. Tumors grew slowly in the other two immunotoxin-treated mice. Control groups of mice (treatment with saline alone, gelonin alone, or HuM195 admixed with rGel at the same final concentrations) did not show significant tumor inhibition or any cures.

To assess whether activity could be observed against larger tumors, in a second trial, we also tested six injections of HuM195-rGel (twice a week for 3 weeks at the same dose of 36 μg per mouse at 14 days and 28 days after transplantation with HL60 cells. Despite the increase in the number of doses from three to six, the delay in treatment to 28 days caused less inhibition of tumor growth by HuM195-rGel (Table 1 and Figure 4b). After 3 weeks of treatment with HuM195-rGel, two out of four mice had no tumors in the group treated 14 days after transplantation; however, all four mice developed local

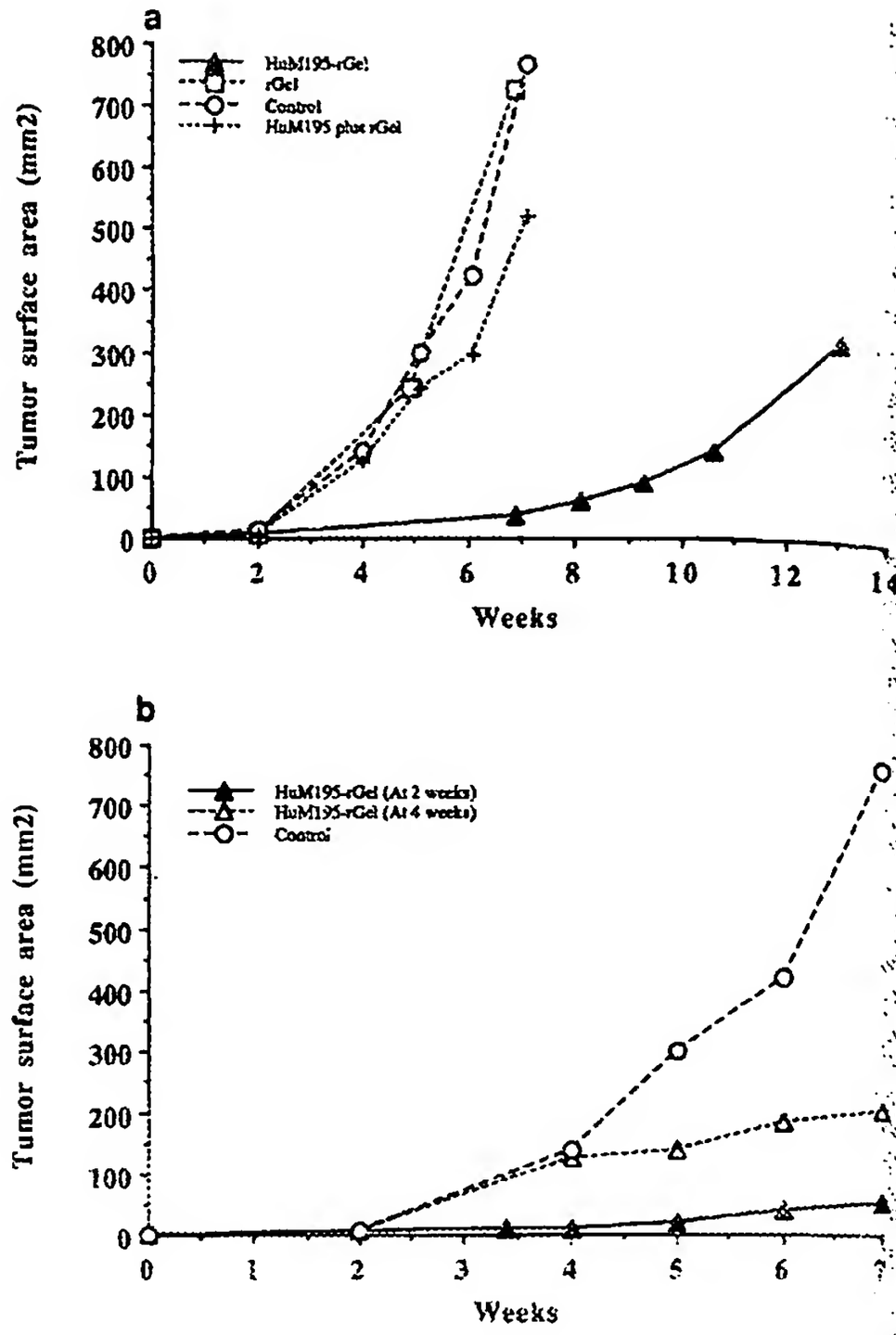

Figure 4 Treatment of human leukemia cells *in vivo* model by HuM195-recombinant gelonin (rGel). Mice were ip transplanted (0) HL60 human leukemia cells as described under Materials and methods. (a) At the tenth day, mice were treated by three injections of 100 nM: HuM195-rGel (four mice); rGel (four mice); HuM195 mixed with rGel (five mice); or control saline (five mice). At times indicated in x axis, tumor surface area was measured. One of five mice in control group and one of five mice in HuM195 admixed with rGel died in the sixth week. (b) At the 14th or 28th days, mice were treated by six injections of 100 nM: HuM195-rGel (four mice at the 14th day); HuM195-rGel (four mice at 28th day); Control saline (five mice). At times indicated in x axis, tumor surface area was measured.

tumors when treated 28 days after transplantation. This may be due to difficulty in delivering HuM195 to larger solid tumors or to the development of resistant cells within the larger tumors.

Discussion

Leukemia-specific immunotoxins (IT) have been shown to exhibit potent cytotoxic activity *in vitro*.[12,14,18,27,28] In this study we have used HuM195-rGel directed to human CD33 antigen to show anti-leukemic effects *in vivo* in a nude mouse model of human leukemia. Anti-tumor effects have been seen in models of lymphoid neoplasms[29–32] and in humans,[33–35] but this is the first report of *in vivo* activity of an IT in myeloid leukemia. CD33 is a useful target antigen for therapy of myelogenous leukemias, as it is expressed on the cell surface of greater than 80% of leukemia isolates from patients with myeloid leukemia and on leukemia colony-forming cells with

Table 1 Tumor size at 7 weeks after HuM195-rGel treatment

Groups	Control	rGel	HuM195 + rGel[b]	HuM195-rGel		
				(at 10 days)	(at 14 days)[a]	(at 28 days)[a]
Mouse 1	420	648	342	No tumor	No tumor	96
Mouse 2	525	696	400	No tumor	No tumor	160
Mouse 3	900	760	550	49	81	180
Mouse 4	1225	803	784	96	117	380
Mouse 5	Death at 6 weeks	N/A[c]	Death at 6 weeks	N/A	N/A	N/A
Mean ± s.d.	768 ± 368	727 ± 68	519 ± 197	36 ± 46	50 ± 59	204 ± 123

Ten million HL60 leukemia cells were transplanted ip into nude mice. Mice were treated with three injections of equimolar amounts of HuM195-rGel, rGel, or HuM195 plus rGel, beginning at 10 days after HL60 transplantation. Two other groups of mice were treated with six injections of HuM195-rGel, beginning at the 14 or 28 days after HL60 transplantation. Subcutaneous tumor size (cross product in mm^2) at 7 weeks is shown.

[a]These mice received six injections instead of three injections.

[b]Injected together, but not conjugated to each other.

[c]N/A, these groups had four mice only.

an average antigen density of 10 000 sites per cell.[16] In addition, rapid internalization occurs upon binding of mAb to CD33 both *in vitro* and *in vivo*.[19,23] HuM195, a humanized version of M195, constructed by genetically grafting the murine complementarity-determining region (CDR) to a human IgG1 framework and constant regions, is reactive with CD33.[19] The humanized antibodies may be advantageous due to reduced immunogenicity, higher avidity, and longer serum half lives.[19,20]

Human CD33 is not expressed on normal mouse tissues. The distribution of CD33 positive cells on human normal tissues is likely to have an important impact on the clinical utility of this immunotoxin. The expression of CD33 antigen in humans is restricted to a small fraction of hematopoietic cells, including colony forming unit-granulocyte monocytes (CFU-GM), some burst forming units-erythroid (BFU-E), a fraction of more primitive progenitors,[13,15,16,24] monocytes, and blood dendritic cells. However, CD33 is not found on tissues outside the hematopoietic system, nor on the normal pluripotent hematopoietic progenitor stem cells.

Major obstacles to the effective therapeutic use of IT in humans include immunogenicity of the IT, toxicity to cells that non-specifically internalize the IT, and difficulty in delivering sufficient IT to tumor sites.[1,33,36] HuM195-rGel may bypass some of these difficulties by the use of a CDR-grafted non-immunogenic mAb that has already demonstrated efficient, specific, and saturable targeting to leukemia cells in humans *in vivo*. In this paper we now demonstrate that the HuM195-rGel will target and internalize into human leukemia cells *in vivo* in a murine model, and subsequently will eliminate or slow tumor growth.

It is possible that conjugation of the mAb to this smaller, less toxic RIP, will provide an immunotoxin that is less immunogenic and more tolerable in humans. The ID_{50} of HuM195-rGel and free rGel *in vitro* was 400 to 1000-fold different. Therefore, some dissociation of the HuM195-rGel bond *in vivo* is not likely to affect the specificity of the treatment; nor should there be significant additional cytotoxic effects on CD33-negative normal cells.

Other investigators have characterized immunotoxins composed of anti-CD33 mAb and ricin A chains *in vitro*. La Russa *et al*[18] and Roy *et al*[27] developed an anti CD33-IT, MY9-blocked ricin (MY9-bR), which also demonstrates potent and selective cytotoxicity towards CD33 positive cells. In the blocked ricin system, non-specific binding of the ricin B chain was blocked by chemically modifying the galactose binding domain. MY9-bR has demonstrated selective inhibition of greater than 85% of the CD33-positive CFU-GM clonogenic growth while sparing the CD34-positive/CD33-negative hematopoietic stem cell. The use of MY9-bR is under investigation as a purging agent prior to autologous bone marrow reinfusion.[37]

HuM195-rGel conjugates showed specific and potent cytotoxicity for CD33 positive leukemia cells *in vivo*. Under the conditions of this model, where HL60 cells grow in the perineum and subcutaneous space of nude mice as a solid tumor, the HuM195-rGel was still effective at inhibiting tumor growth. Importantly, the early treatment of leukemia tumors with HuM195-rGel was able to cure half of the mice that had already developed local tumors. Although the leukemic tumor size at 10 days is only about 2 mm^2, this represents about 10^7 cells in the subcutaneous tumor site. Although cell numbers are much larger in humans, leukemia cells are more dispersed, allowing for even more rapid penetration and possibly greater efficacy. At later time-points after transplantation into the mice, the HuM195-rGel therapy was less effective at these dose levels, suggesting that immunotoxin penetration into the larger tumors (10^8–10^9 cells) was not adequate or that resistant cell populations may have developed. These data suggest that HuM195-rGel is a potent and specific cytotoxic agent active against cells expressing a leukemia-restricted antigen. In addition, these data suggest that treatment of human leukemia with HuM195-rGel immunotoxin is more likely to be effective in the setting of minimal leukemic disease, such as after chemotherapy or in the setting of bone marrow purging.

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Characterization of murine and humanized anti-CD33, gelonin immunotoxins reactive against myeloid leukemias

Kimberly J. McGraw[1], Michael G. Rosenblum[2], Lawrence Cheung[2], David A. Scheinberg[1]

[1] Memorial Sloan Kettering Cancer Center, New York, NY 10021, USA
[2] Immunopharmacology Section, Box 41, Department of Clinical Immunology and Biological Therapy, M. D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030, USA

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Abstract. M195 antibodies recognize CD33, an antigen present on acute myeloid leukemia blasts as well as some myeloid progenitor cells, but not on the ultimate hematopoietic progenitor stem cell. Immunotoxins (IT) reactive with human myeloid leukemias were constructed by conjugating gelonin, a single-chain ribosome-inactivating protein, to murine and genetically engineered, humanized M195 antibodies via an *N*-succinimidyl-3-(2-pyridyldithio)-propionate linkage. No losses of gelonin cytotoxic activity or M195 binding activity were observed after conjugation of up to two toxin molecules per antibody. Toxin conjugates displayed specific, potent toxicity for CD33+ cells. The murine and humanized IT were not toxic to CD33− cells and were 600 and 4500 times more potent, respectively, than free gelonin in inhibiting CD33+ HL60 cells. Treatment of HL60 cells with 1 μg/ml HuM195-gelonin resulted in more than 1000 times lower colony formation; normal bone marrow mononuclear cell colony-forming units treated with HuM195-IT were reduced by a factor of 10. HL60 leukemia cells could be effectively purged from an excess of normal bone marrow cells. Exposure of target cells to IT for as little as 30 min was as effective as continuous exposure of IT for up to 6 days. However, measures of the efficacy of the immunotoxin were directly related to the length of time of observation after IT exposure and were inversely related to cell concentration. M195-gelonin immunoconjugates are potential candidates for therapeutic use in in vivo or ex vivo bone marrow purging of myeloid leukemias.

Key words: Myeloid leukemia – CD33 – Immunotoxin – Gelonin – M195 – HuM195

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Correspondence to: D. A. Scheinberg

Introduction

Immunotoxins (IT) are a class of proteins that consist of a monoclonal antibody (mAb) covalently linked or genetically fused to a toxic molecule and are thus able to direct potent cytotoxicity to particular cells [41, 22]. Obstacles to effective therapeutic use of immunotoxins for cancer include (a) lack of suitable tumor-specific targets that are not also found on other vital non-tumor cells [39]; (b) loss of toxin potency or mAb activity after conjugation [15]; (c) unwanted cytotoxicity to nontarget cells and tissues resulting from nonspecific internalization of the IT [22]; (d) immunogenicity of the IT [10, 21, 27]; (e) pharmacological inability to target tumor sites adequately [10, 20].

CD33 provides a useful target antigen for therapy of myelogenous leukemias, as it is expressed on the cell surface of more than 80% of leukemia isolates from patients with myeloid leukemia with an average antigen density of 10000 sites/cell [1, 18, 32, 38]. In addition, rapid internalization occurs upon binding of mAb to CD33 both in vitro and in vivo [14, 33]. CD33 is also found on normal granulocyte/monocyte-colony-forming units (CFU-GM), some burst-forming colonies and a fraction of the more primitive progenitors [1, 18, 32, 38]. However, CD33 is not found on tissues outside of the hematopoietic system nor on the normal pluripotent hematopoietic progenitor stem cell. My9, a murine IgG2B mAb reactive with CD33, has been conjugated to a chemically modified ricin toxin and demonstrates potent specific cytotoxicity in vitro [23, 31]. Both M195, a murine monoclonal IgG2a antibody, and HuM195, a humanized version of M195 constructed by genetically grafting the murine CDR regions to a human IgG1 framework and constant regions, are reactive with CD33 [8, 12, 33, 38]. The humanized antibodies may be advantageous because of their reduced immunogenicity, higher avidity, and longer serum half lives [7, 8, 12].

Both murine and humanized M195 are now in clinical trials for the treatment of myelogenous leukemias. ^{131}I-radiolabeled M195 is capable of killing up to 10^{12} leukemia cells in patients with refractory or relapsed leukemias [35]. However, because of the long-range cytotoxicity of the

conjugated nuclide (approximately 50 cell diameters), killing of normal bystander cells occurs as well, requiring bone marrow transplantation at high doses. Use of an anti-CD33 IT may avoid this problem by killing only those cells bearing the CD33 antigen.

Gelonin is a 30-kDa single chain ribosome-inactivating protein isolated from the seeds of *Gelonium multiflorum*, which irreversibly inactivates the ribosomal 60S subunit by cleaving the *N*-glycoside bond of adenine in a specific sequence of rRNA and thereby prevents protein elongation [2, 37]. Gelonin has several advantages over other ribosome-inactivating proteins currently being investigated. Unlike the dual-chain type, such as ricin [4] and abrin [36], which contain a B chain reactive with cell-surface carbohydrates, gelonin is a biochemically stable single chain [22, 29, 36]. It lacks a galactose-specific lectin domain, which is responsible for non-specific binding and toxicity to cells. As a result, free gelonin is much less toxic to intact mammalian cells in vitro and in vivo than the heterodimeric toxins. Despite this relative safety, in a cell-free rabbit reticulocyte translation assay, gelonin demonstrates nearly equal activity to heterodimeric toxins [6, 26]. Attempts have been made to prevent the non-specific binding of the B chain of heterodimeric toxins by separating the A and B chains or by blocking of galactose-binding sites [21, 31]. These methods are technically difficult, variably effective, or sometimes incomplete; hence the IT may contain trace contamination with active B chain that can result in a less selective immunoconjugate and potential toxicity in vivo. Residual membrane-binding activity of the ricin B chain may be necessary for translocation of the A chain [17]. In addition, the larger heterodimers may also be more immunogenic than the single-chain toxins.

Thus, gelonin presents a potential advantage over the dual-chain toxins and was selected for this study. In this paper, we describe immunotoxins constructed by chemical conjugation of mAb reactive against CD33 antigen with the plant toxin gelonin.

Materials and methods

Cell culture. Cell lines HL60 (acute myeloid leukemia, CD33+), U937 (monocytic leukemia, CD33+), Raji and Daudi (B lineage Burkitt's lymphomas, CD33-), Molt4 (T lineage lymphoma, CD33-) and SKLY16 (B lineage lymphoma, CD33-), were maintained in culture using RPMI-1640 medium supplemented with 10% Serum Plus (JRH Biosciences), 5% heat-inactivated newborn bovine serum (Armour Pharmaceuticals), non-essential amino acids, penicillin and streptomycin.

Transformed murine fibroblast cell lines NIH-3T3 and AL67 [24], the latter expressing the transfected CD33 gene, were maintained in culture similarly.

Antibodies. mab M195 and HuM195 were prepared as described [8, 32, 38]. Highly purified HuM195 and HuG1 Fd79 [11], a genetically engineered human IgG1 construct reactive with a herpes simplex virus antigen not found on HL60 cells, were the generous gifts of Man Sung Co, Protein Design Labs, Mountain View, Calif.

SPDP conjugation. A threefold molar excess of *N*-succinimidyl 3-(2-pyridyldithio)-propionate (SPDP), prepared in dry dimethyl-formamide, was added to 10 mg M195 or HuM195 in phosphate-buffered saline pH 7.4 (PBS) and incubated for 30 min at room temperature. Excess SPDP was removed on a Sephadex G-25 column equilibrated with 0.1 M phosphate buffer pH 7.0 containing 0.5 mM EDTA.

Modification of gelonin with 2-Iminothiolane. 2-Iminothiolane in 0.5 M triethanolamine hydrochloride (TEA/HCl), pH 8.0, was added to 10 mg gelonin in 60 mM TEA/HCl, 1 mM EDTA, pH 8.0, and incubated for 90 min at 4 °C under nitrogen. Excess 2-iminothiolane was removed by gel filtration on a Sephadex G-25 column equilibrated with 5 mM BISTRIS/acetate buffer pH 5.8 containing 50 mM NaCl and 1 mM EDTA.

Conjugation of M195 with gelonin

SPDP-modified M195 in 100 mM sodium phosphate buffer, 0.5 mM EDTA, pH 7.0, was mixed with a five-molar excess of 2-iminothiolane-modified gelonin. The pH was adjusted to 7.0 with 0.5 M TEA/HCl, pH 8.0, and the mixture was incubated for 20 h at 4 °C under nitrogen. To stop the reaction, iodoacetamide was added to a final concentration of 2 mM and incubated for 1 h at room temperature.

Purification. The reaction mixture was filtered on a Sephacryl S-300 gel filtration column equilibrated with 20 mM TRIS, 50 mM NaCl, pH 7.4, to separate the antibody and antibody-gelonin conjugates from the free gelonin. The fractions containing immunotoxin and unreacted antibody were pooled and then loaded on a Cibacron-blue–Sepharose CL-6B column equilibrated with TRIS buffer to remove the unconjugated antibody. Purified immunotoxin (M195-IT) was eluted with 20 mM TRIS buffer containing 2 M NaCl, pH 7.4. Collected fractions were dialyzed against PBS.

Flow-cytometry assays. Cells were washed and resuspended in RPMI-1640 medium, with 10% Serum Plus, non-essential amino acids, penicillin, streptomycin, and 2% rabbit serum (Pel Freeze). Samples containing 500000 cells in a final volume of 0.15 ml were incubated for 1 h on ice in the presence of primary antibody. Cells were washed twice, incubated for 1 h on ice with secondary fluorescein-isothiocyanate(FITC)-labelled antibody [goat anti-(mouse Ig) or goat anti-(human Ig)], washed twice, and fixed with 0.5% paraformaldehyde. The FITC fluorescence intensity was measured on an EPICS Profile II flow cytometer [32].

Enzyme-linked immunosorbent assay (ELISA). Primary antibody was added to 96-well plates containing adherent AL67 or NIH-3T3 cells and allowed to bind for 1 h at room temperature. CD33 is overexpressed and is not modulated quickly off of the surface of AL67 cells. Excess antibody was removed by washing the cells three times. Alkaline-phosphatase-labelled secondary antibody was added and allowed to incubate for 1 h at room temperature. Cells were washed three times and 100 µl substrate solution *p*-nitrophenyl disodium phosphate was added. Absorbance at 405 nm was measured using a Fisher Biotek microplate reader after a 10- to 20-min incubation at 37 °C.

Rabbit reticulocyte lysate translation assay. The functional activity of gelonin and gelonin-containing immunotoxin was assayed by a cell-free translation inhibition assay kit (Gibco-BRL) as described by the manufacturer.

Inhibition of [^{3}H]thymidine, [^{3}H]leucine, and ^{3}H-labelled amino acid incorporation. Samples containing 100 µl cells were washed and incubated at 37 °C in 96-well plates in the presence of 50 µl antibody, conjugate, or toxin. After an incubation time of 3–7 days, 50 µl 10 µCi/ml tritiated thymidine, leucine, or amino acids was added to each well and allowed to incorporate for 5–6 h. A 50-µl aliquot of trichloroacetic acid was added to precipitate protein for [^{3}H]-leucine and ^{3}H-labelled amino acid incorporation experiments. Cells were harvested with a Skatron semiautomatic harvester and assayed in a Packard scintillation counter.

3-[4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide (thiazoyl blue; MTT) assays. Samples containing 100 µl cells were washed and incubated at 37 °C in 96-well plates in the presence of 50 µl antibody, conjugate, or toxin. After an incubation time of 3–7 days, the plate was centrifuged for 5 min at 1000 rpm. MTT, diluted in PBS, was added to each well and incubated for 4 h. Plates were washed. The formazan product was solubilized with 0.04 M HCl in propan-2-ol and quantified spectrophotometrically at 570 nm.

Competition assays. HL60 cells at 1×10^5 cells/ml were incubated for 1 h on ice in the presence of excess HuM195 or HuFd79. HuM195-IT, at a concentration capable of killing approximately 70% of cells, was then added. Cells were incubated for 90 h at 37 °C then assayed by trypan blue exclusion or [^{3}H]thymidine incorporation.

Time course studies. HL60 cells at 0.67×10^5 cells/ml were incubated with HuM195-IT. At various times cells wee washed twice and re-suspended in an IT-free medium. On day 6 the cells were plated onto a 96-well plate and analyzed by trypan blue exclusion or [^{3}H]thymidine incorporation.

Clonogenic growth. HL60 cells were treated with HuM195-IT and allowed to incubate for 24 h at 37 °C. Cells were then washed and plated in 1 ml 0.3% agarose, RPMI-1640 medium, 8.33% newborn bovine serum, 18.33% fetal calf serum (FCS), non-essential amino acids, penicillin and streptomycin. Plates were incubated for 14 days at 37 °C.

Fourteen-day CFU-GM. Fourteen-day CFU-GM assays were performed essentially as described [16] using various cell concentrations plated onto 1-ml agarose dishes supplemented with granulocyte-colony-stimulating factor, granulocyte/macrophage-colony-stimulating factor and interleukin-3. Growth factors were the generous gift of Janice Gabrilove, MSKCC. Assays were done in duplicate or quadruplicate and each experiment was repeated three times. The plating efficiency was approximately 0.1%.

Purging of HL60 from excess normal bone marrow. Bone marrow aspirates were obtained from normal donors according to Memorial Sloan Kettering Cancer Center IRB protocols. Mononuclear cells were collected by Ficoll-Paque sedimentation, washed, and gamma-irradiated with 8 Gy. Marrow cells were divided into aliquots in 96-well plates at a final concentration of 1×10^6 cells/ml. HL60 cells at a final concentration of 0.667×10^5 cells/ml and HuM195-IT at various concentrations were added to the plates. After a 6-day incubation at 37 °C, cells were assayed for [^{3}H]thymidine incorporation.

Results

Conjugation and purification

Both M195 and HuM195 were conjugated with gelonin and purified as described in the Materials and methods. As shown in Fig. 1, purified M195 antibody migrates on the sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) as three major protein bands representing the glycosylated and carboxyterminal-modified forms of M195 [13]. The final purified conjugate was also found to contain three major protein bands as shown in lane C. Since, electrophoretic analysis alone could not confirm whether the final purified immunotoxin contained any unconjugated antibody, analysis of the final immunotoxin preparation was done by Western blot analysis using anti-gelonin rabbit polyclonal antisera to confirm the presence of gelonin in each of the major Coomassie-stained bands (data not shown).

Fig. 1. Sodium dodecyl sulfate gel electrophoretic fractionation of M195 and the immunotoxin M195-IT under non-reducing conditions with a gradient of 5%–20% acrylamide. *Lanes from left to right:* purified mouse M195; reaction mixture containing M195 and gelonin; purified M195-gelonin immunotoxin; purified gelonin

Binding specificity and titer of IT

The binding specificities of M195-IT and HuM195-IT were tested against a variety of CD33+ (U937, HL60, AL67) and CD33- (SKLY16, Raji, Molt4, Daudi) cell lines using flow cytometry or ELISA. The conjugates retained the same specificity for CD33-expressing cells as the unconjugated antibodies.

Different batches of conjugates containing approximately one or two gelonin molecules per mAb showed no loss of binding titer as compared to unconjugated HuM195 (Fig. 2A, B) or M195 (data not shown). However, a batch of HuM195 conjugated with an average of three gelonin molecules per mAb, as determined by SDS-PAGE, demonstrated a lower binding titer against both HL60 cells, as assayed by indirect flow cytometry, and AL67 cells, as assayed by ELISA (data not shown). Therefore, all additional experiments presented in this paper used batches of gelonin conjugated to antibody at a ratio of approximately 1:1 unless otherwise stated.

Biological activity of toxin

The ability of HuM195-IT and gelonin to inhibit translation in a cell-free system was assayed using a rabbit reticulocyte lysate translation assay. Both the immunotoxin and free gelonin demonstrated a similar 50% inhibitory concentration (ID_{50}) in the range of 12–16 pM, demonstrating that conjugation of gelonin to the antibody did not alter the activity of the toxin (Fig. 3).

Cytotoxicity of immunoconjugates. Both M195-IT and HuM195-IT were tested for their ability to kill CD33+ and CD33- cells in comparison to free gelonin. Cytotoxicity was determined by a variety of methods including inhibition of incorporation of tritiated amino acids into trichloroacetic-acid-precipitable protein, inhibition of DNA synthesis, trypan blue exclusion, MTT, and clonogenic assays (not all shown). M195-IT had an ID_{50} of approximately 400 pM, which was approximately 600 times more potent than free gelonin (Fig. 4). HuM195-IT, which has a higher affinity for CD33 than M195, had an ID_{50} of 15 pM, which was 4500 times more potent than the ID_{50} of free gelonin (Fig. 5). Different lots of HuM195-IT displayed

370

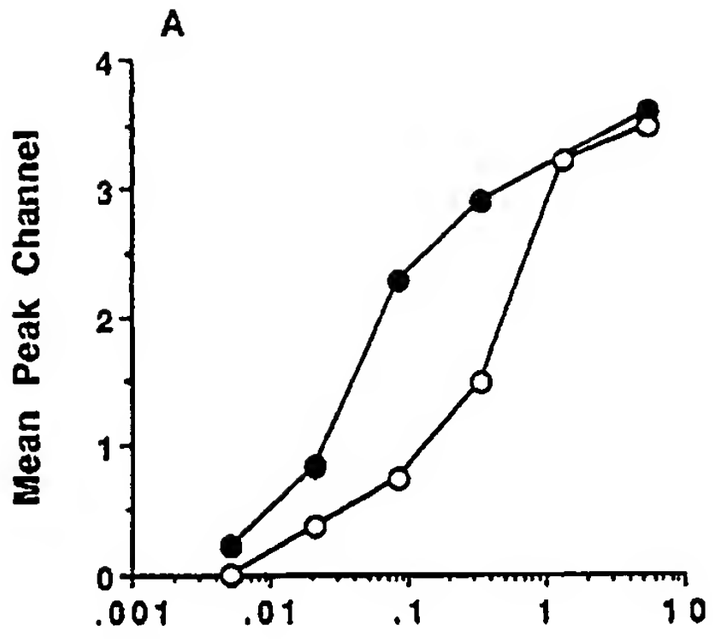

Fig. 2A, B. Binding titer and specificity of HuM195-IT on cell lines. HL60, U937, or Molt4 cells at a final concentration of 1.5×10^6 cells/ml were incubated on ice for 1 h with either HuG1 M195 or HuG1 M195-IT a a final concentration range of 0.08 µg/ml to 10 µg/ml. Mean peak fluorescence intensity (*y* axis) versus mAb or immunotoxin (IT) concentration (*x* axis) was measured using an EPICS Profile II cytometer. A HL60 binding by HuM195-IT (●) or by HuM195 (○). B U937 binding by HuM195-IT (●) or by HuM195 (○); Molt4 binding by HuM195-IT (■) or by HuM195 (△)

different levels of cytotoxicity with some lots having an ID_{70} of 5 pM. There was a slow loss of potency over time (months) suggesting either reduction of the intermolecular linkage or denaturation of the IT. Because of the variabilities, accurate comparisons of potency between the murine and humanized conjugates are not possible.

Cytotoxicity, as determined by ^{3}H-labelled amino acid or [^{3}H]thymidine incorporation, was confirmed to result in cell death by examining parallel wells by trypan blue analysis or similarly treated cells as measured by MTT (not shown).

Non-specific cytotoxicity was not observed with the IT. Even at the highest concentration of immunotoxin used, typically 10–16 µg/ml, no cytotoxicity was observed when either conjugate was incubated with the CD33-negative cell lines Raji, Molt4, Daudi, and SKLY16 (not shown).

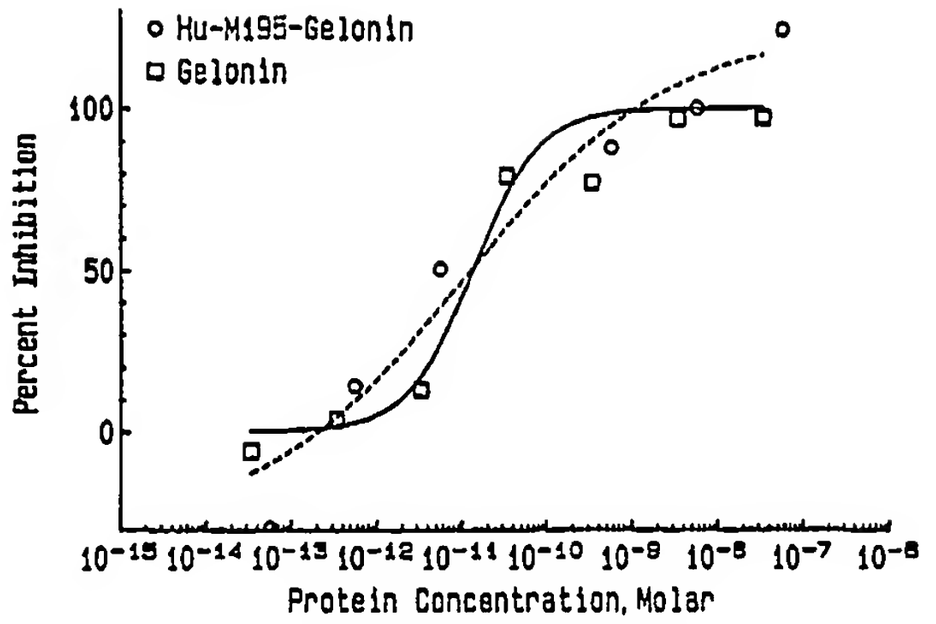

Fig. 3. Rabbit reticulocyte lysate translation assay. Increasing amounts of free gelonin toxin (□) or HuM195-IT (○) were assayed for activity in a cell-free lysate translation inhibition assay. HuM195-IT final concentrations ranged from 5 pg/ml to 5 µg/ml. Gelonin final concentration ranged from 1 pg/ml to 1 µg/ml

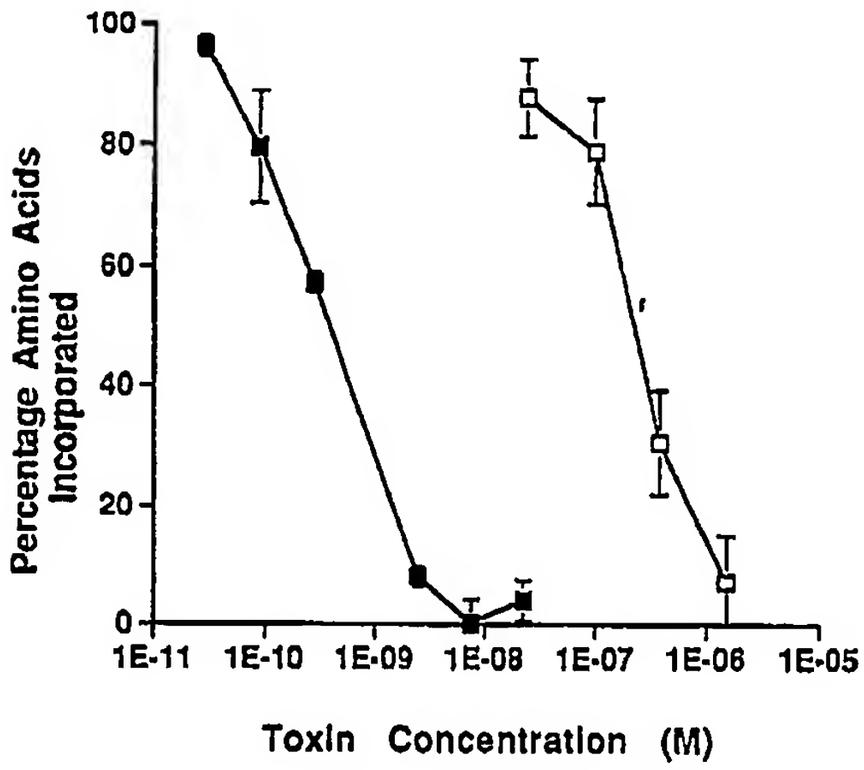

Fig. 4. Inhibition of protein synthesis in live cells by gelonin and M195-IT on HL60 cells. HL60 cells at a final concentration of 1×10^6 cells/ml were incubated for 3 days at 37 °C in the presence of M195-IT (■) and gelonin (□). Levels of protein synthesis were determined by 5 h incorporation of tritiated amino acids into trichoroacetic-acid-precipitable protein. M195-IT final concentrations ranged from 5 ng/ml to 4 µg/ml. Gelonin final concentration ranged from 0.5 µg/ml to 50 µg/ml. The data are representative of four experiments

Because of the increased potency shown above and the theoretical advantage of reduced immunogenicity of the humanized form of the M195 in vivo, all further experiments were conducted using the HuM195-IT.

HuM195-IT inhibited the clonogenic growth of HL60 in a dose-dependent manner. Incubation of cells with 10 µg/ml and 1 µg/ml HuM195-IT for 72 h decreased colony formation from 8225 colonies/10^5 cells plated to 5 and 13 colonies/10^5 cells plated respectively (Fig. 6). Incubation with 0.1 µg/ml HuM195-IT and unconjugated antibody did not significantly inhibit growth.

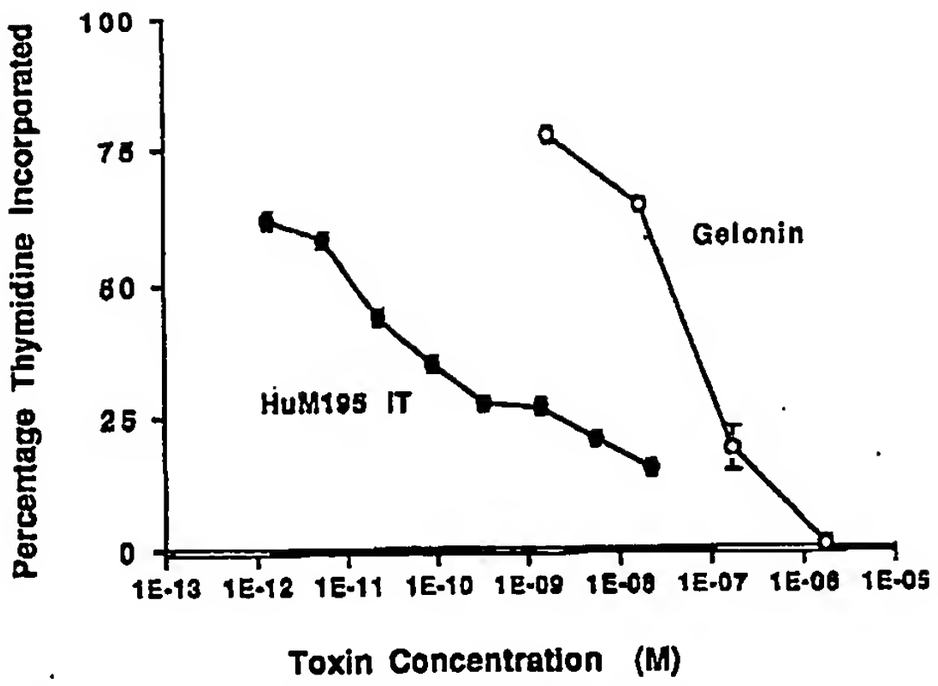

Fig. 5. Inhibition of DNA synthesis in live cells by gelonin and HuG1 M195-IT on HL60 lines. HL60 cells at a final concentration of 3×10^4 cells/ml were incubated for 5 days at 37 °C in the presence of HuG1 M195-IT (●) or gelonin (○). Levels of DNA synthesis were determined by 5 h incorporation of tritiated thymidine. HuG1 M195-IT final concentrations ranged from 0.2 ng/ml to 4 μg/ml. Gelonin final concentration ranged from 0.5 μg/ml to 50 μg/ml. The data are representative of five experiments

HuM195-IT at 0.25 μg/ml, 1 μg/ml and 10 μg/ml incubated with bone marrow mononuclear cells resulted in 60%–90% decreases in colony-forming units. Gelonin at the same molar concentration as 1 μg/ml IT resulted in an approximately 70% decrease in colony-forming units.

Specificity for CD33 sites

To confirm further the specificity of cytotoxicity of the HuM195-IT for $CD33^+$ cells and its requirement for binding to the CD33 antigen itself, HL60 cells were incubated on ice in the presence of an excess of unconjugated HuM195 or HuG1Fd79, an isotype control antibody. Preincubation of HL60 cells with 50 μg/ml HuM195 was able to inhibit more than 85% of the HuM195-IT cytotoxic activity, whereas HuG1Fd79 did not block killing. This confirmed that specific targeting of leukemic cells was through the CD33-antigen-binding site and not through the Fc region or other non-specific binding sites on target cells.

Effects of target cell concentration

In order to determine the effect that cell concentration may have upon the efficacy of the HuM195-IT, HL60 cells were serially diluted and incubated in the presence of a single concentration of IT at 2 μg/ml. The immunotoxin was most effective at low cell concentrations (Fig. 7). At higher cell concentrations, the IT lost potency. This was not due to an excess of cell surface binding sites over IT molecules as, even at the highest cell concentrations, there were 100–1000 more molecules of IT than available binding sites. In other experiments, serial dilutions of IT and gelonin were incubated with three different concentrations of HL60 cells. Both the IT and gelonin were more potent at lower cell concentrations (data not shown). Experiments looking for transferable cell-free inhibitory factors secreted or released by target cells in high concentrations were negative (not shown).

Killing of leukemia targets contaminating normal bone marrow

The dependence of killing on cell density raised the issue of the efficacy of the IT in the presence of large numbers of non-target cells. Therefore, to determine whether the IT was able to kill HL60 cells in the presence of excess numbers of $CD33^-$ cells, HL60 cells were mixed with a 15-times excess of irradiated normal bone marrow cells. This ratio simulates that which might be found in a typical marrow in early relapse contaminated with low levels of leukemia cells. The presence of the bone marrow had minimal effect upon the cytotoxicity of the IT (Fig. 8).

Effects of length of exposure of IT with target cells. CD33 modulation and internalization begin within minutes of mAb binding in vitro and in vivo [14, 33, 38]. HL60 cells were incubated in the presence of HuM195-gelonin at 1 μg/ml for 30 min, 1 h, 4 h, and 6 days and then washed twice to

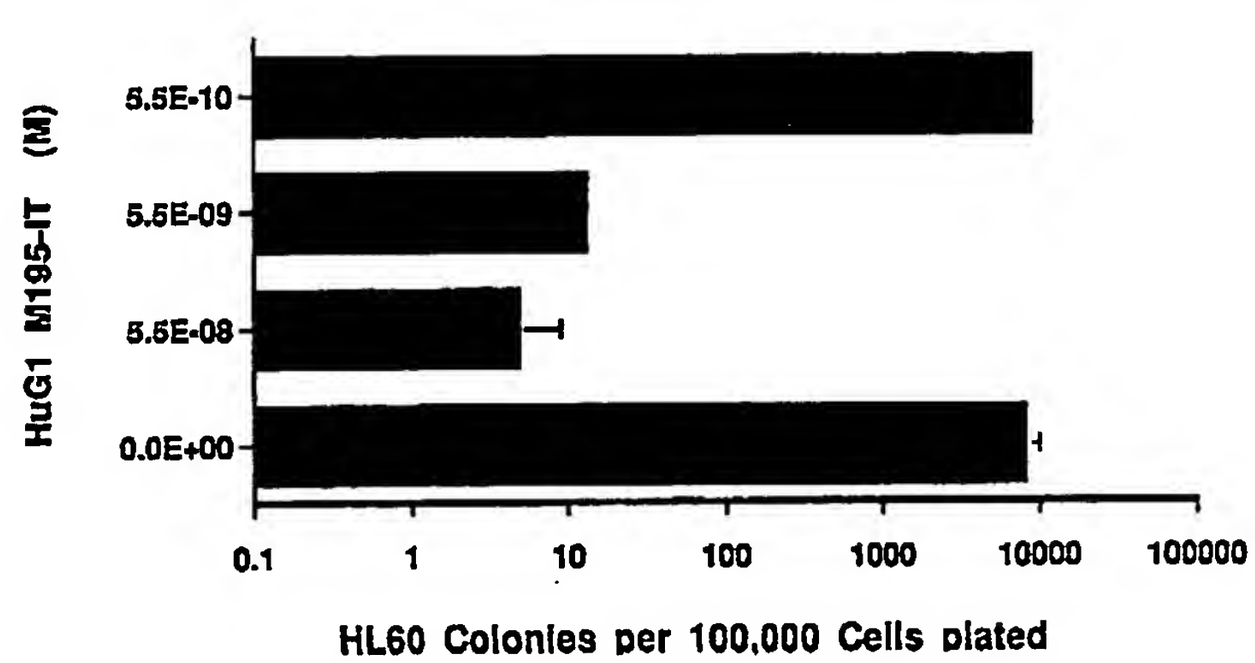

Fig. 6. Effect of HuG1 M195 upon HL60 colony formation. HL60 cells at 6.67×10^4 cells/ml were incubated in the presence of various concentrations of HuG1 M195 for 3 days. Cells were then washed and plated in 0.36% agarose, 20% fetal calf serum, 10% Serum Plus. Colonies were assayed after a 14-day incubation. The data are representative of two experiments

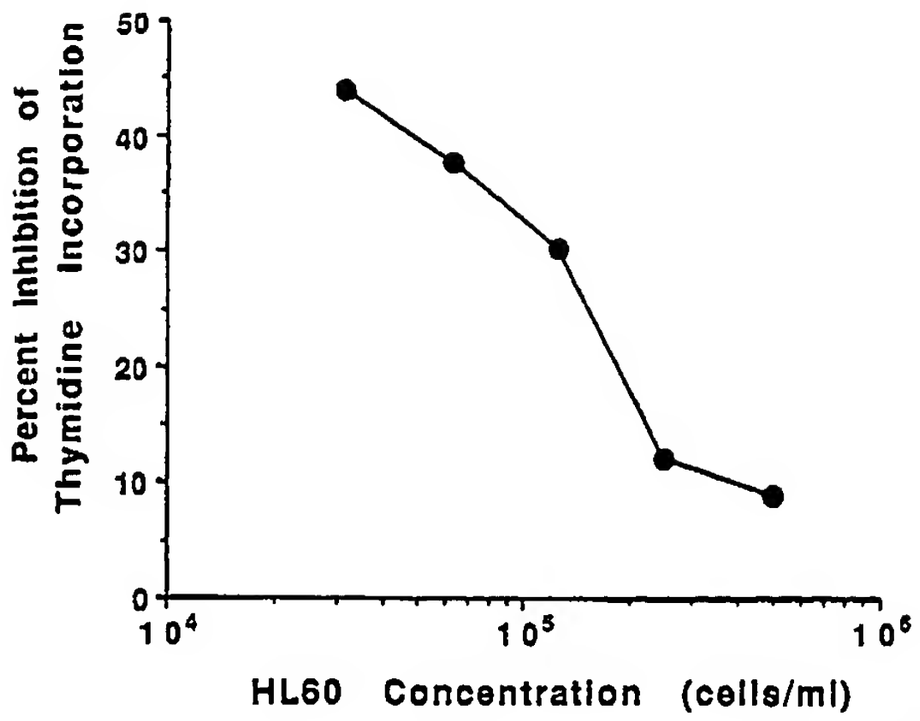

Fig. 7. Effect of HL60 concentration upon efficacy of HuM195-IT. HL60 cells at a final concentration of 3.125×10^4–5×10^5 cells/ml were incubated for 5 days at 37 °C with or without IT at a final concentration of 2 μg/ml. DNA synthesis was determined by 5 h incubation with tritiated thymidine. Percentage inhibition was determined in comparison to control wells without IT. The data are representative of three experiments

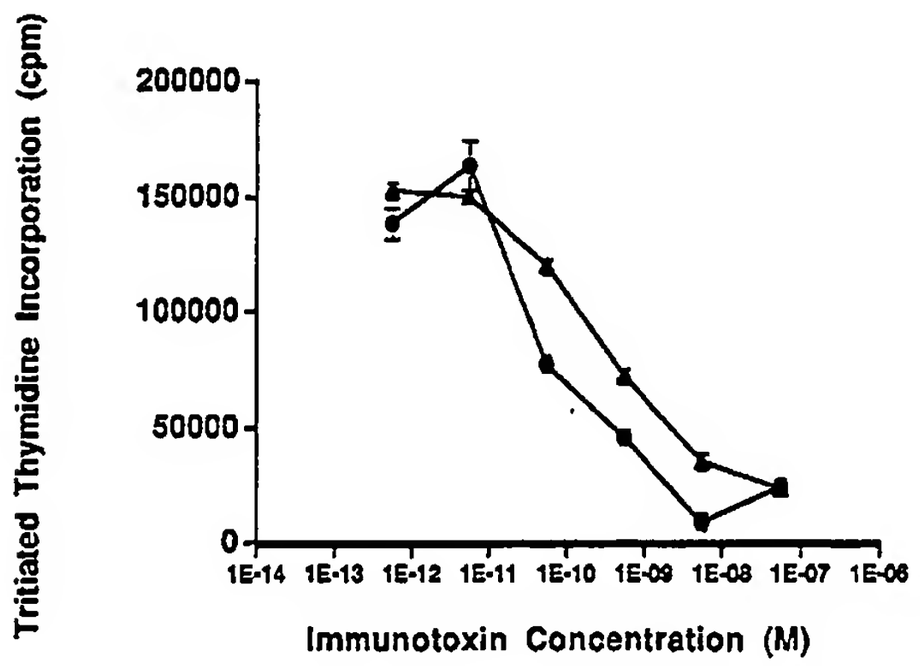

Fig. 8. Cytotoxicity of HuM195-IT on HL60 in the presence of excess irradiated bone marrow. HL60 cells at a final concentration of 6.67×10^4 cells/ml in the presence (▲) or absence (●) of normal irradiated bone marrow at 1×10^6 cells/ml were incubated for 6 days at 37 °C in the presence of HuG1 M195-IT at a final concentration of 100 pg/ml–10 μg/ml. Levels of DNA synthesis were determined by measuring 5 h tritiated thymidine incorporation. The data are representative of two experiments

remove non-bound immunotoxin. The cells were resuspended in fresh media and returned to 37 °C and incubated for 6 days from the day of initial exposure. On day 6, the cells were analyzed by trypan blue exclusion. No significant differences in cell killing were observed in relationship to the time of exposure to IT (data not shown). This suggested that the critical event leading to toxicity was the initial binding of IT to CD33 sites, and that this occurred within 30 min.

Following exposure, the length of time necessary to observe cell killing was also studied. HL60 cells with various concentrations of HuM195-IT were harvested after 3, 4, and 5 days of exposure. The median lethal dose (LD_{50}) at 5 days (0.3 nM) was 30 times lower than the LD_{50} at 4 days (8.9 nM) and 40 times lower than the LD_{50} at 3 days (12 nM). Therefore, although the initial event necessary for cytotoxicity occurred within 30 min, the subsequent events leading to cell death required considerable time to develop fully.

Discussion

Major obstacles to the effective therapeutic use of IT in humans include immunogenicity of the IT, toxicity to cells that non-specifically internalize the IT, and difficulty in delivering sufficient IT to tumor sites [10, 15, 19, 20–22, 39, 41]. The HuM195-gelonin immunotoxin may bypass some of these difficulties by use of a CDR-grafted non-immunogenic mAb that has already demonstrated rapid, efficient, specific, and saturable targeting to leukemia cells in humans in vivo, and by conjugation of that mAb to a smaller, less toxic RIP.

M195-gelonin conjugates showed specific and potent cytotoxicity for CD33+ leukemia cells, required short exposure times for activity, and were unaffected by the presence of excess irradiated bone marrow. The immunotoxin conjugates containing fewer than three gelonin molecules per antibody maintained complete biological activity of the mAb, as measured in flow-cytometric assays, and toxin activity as measured in a rabbit reticulocyte lysate translation assay. Conjugates with more than two gelonin molecules had a decreased avidity for the antigen, which resulted in a less potent molecule. The loss of avidity may be due to stearic interference with the antigen-binding site or to instability of the over-conjugated IT.

The humanized M195-gelonin construct had an ID_{50} of 15 pM, which is approximately 4500 times more potent than free gelonin. When compared to free gelonin, the humanized immunotoxin appeared more potent than the murine immunotoxin; this may be due to the significantly higher affinity of HuM195 than murine M195 for CD33 [8, 12, 13]. Neither construct was toxic towards CD33-negative cell lines at the highest concentrations tested. Specificity analysis and competition assays showed selective binding and activity through CD33 antigen. HL60 clonogenic assays showed that a reduction by more than a factor of 1000 in colony formation was possible with a single treatment of IT.

Murine antibodies usually elicit a human anti-(mouse antibody) response, which results in the rapid clearance of murine mAb from serum and loss of therapeutic efficacy [10, 20, 34, 35]. The genetically engineered humanized antibody HuM195 appears to be largely nonimmunogenic [9]. In addition, the smaller-sized gelonin may be less immunogenic than the larger dual-chained ribosome-inactivating proteins. Therefore, when conjugated to a non-immunogenic humanized mAb a reduction in IT immunogenicity is possible, although clinical trials will be needed to answer this question directly.

Gelonin has several advantages for use in immunotherapy over other ribosome-inactivating proteins including

the lack of the B chain containing the galactose-specific lectin domain responsible for nonspecific binding and toxicity [36, 37]. Despite this, it is equally active in cell-free lysate experiments [6]. Immunotoxins containing an A chain separated from a B chain are in general less potent on intact cells [4, 5]. Therefore, second-generation immunotoxins used high-affinity ligands to block galactose-binding sites [5, 19, 23, 31]. However, complete blocking of the nonspecific galactose residues of the ricin B chain can prevent translocation of b-ricin immunotoxin into cells resulting in a less potent immunotoxin [17].

Recently gelonin toxin has been cloned and expressed in *Escherichia coli*. The recombinant molecule was shown to be as active in the rabbit reticulocyte lysate translation assay as is the native molecule [30]. Thus, recombinant gelonin may have an advantage over native gelonin because of the lack of glycosylation of the recombinant molecule and because eventual fusion toxins of HuM195 with gelonin could result in a more defined molecule that may avoid inadvertent inactivation of either the toxin or the antibody molecule during the chemical conjugation process or during subsequent reduction during storage.

When compared to other toxin-Ab conjugates in murine experiments in vivo, using an anti-melanoma antibody conjugated to gelonin, abrin A, or ricin A, the gelonin-antibody conjugates had advantageous characteristics. Although the gelonin conjugate was not the most potent conjugate in vitro, the gelonin-conjugate IT demonstrated better tumor localization and inhibited tumor growth at levels that demonstrated little toxicity. The other toxin conjugates had no effect upon tumor growth at the maximum tolerated doses [36].

Hematopoietic cancers, and leukemias in particular, are especially suited for antibody-based therapies because of the ability to deliver Ab rapidly and effectively to cells in vivo or for in vitro purging [9, 23, 25, 33, 35, 40]. Clinical trials using ^{131}I-labeled M195 have demonstrated rapid and specific localization of antibody to tumor sites, saturation of all available CD33, followed by intracellular internalization in humans [9, 33]. Dose-escalation studies using ^{131}I-M195 resulted in more than 99% killing of leukemic blasts with negligible toxicity outside of the hematopoietic compartments [35].

CD33 is an unusual leukemia-associated antigen, which is not found on tissues outside the hematopoietic system [1, 18, 32, 38]. Its expression within the hematopoietic system is limited to early myeloid progenitor cells, monocytes and dendritic cells. It is important to note that CD33 is not found on the ultimate hematopoietic progenitor stem cell, thus allowing selective elimination of leukemia cells and early progenitors while preserving capacity for long-term regulation of marrow.

Another anti CD33-directed IT, MY9-blocked ricin (MY9-bR), also demonstrates potent and selective cytotoxicity towards CD33$^+$ cells [23, 31]. In this system the nonspecific binding of the ricin B chain was blocked by chemically modifying the galactose-binding domain. MY9-bR has demonstrated selective inhibition of more than 85% of the CD33$^+$ CFU-GM clonogenic growth while sparing the CD34$^+$/CD33$^-$ hematopoietic stem cell. Because of differences in experimental design, it is difficult to compare results with the M195-IT and My9-IT directly. Differences in their activities may relate to differences in the antibody, the toxin chosen, and the assay systems.

The M195-IT was most effective at killing cells at low cell concentrations, raising questions about its use in patients with fully blastic leukemia. Despite this, HL60 cells were effectively killed even in the presence of excess normal marrow cells. The use of M195-IT may be particularly suited for treatment of residual disease, a conclusion reached by others investigating IT in vivo [19, 21].

HuM195-IT did not completely prevent hematopoietic reconstitution after treatment, as shown by bone marrow colony experiments. Bone marrow treated with HuM195-IT demonstrated a reduction of colony formation by a factor of 10. This is expected since CFU-GM do express CD33 [1, 18, 32]. Regrowth of marrow from earlier progenitors after purging with anti-CD33 agents has been demonstrated by several studies [3, 23, 28]. The reduced toxicity as compared to HL60 may relate to lower levels of CD33 on these cells, decreased internalization of IT, intrinsic resistance of these progenitors, or the type of assay used.

We conclude that M195-gelonin conjugates are highly specific and toxic reagents that may have potential for use in the treatment of myeloid leukemias in vivo or for bone marrow purging ex vivo.

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Anti-MY9-Blocked-Ricin: An Immunotoxin for Selective Targeting of Acute Myeloid Leukemia Cells

By Denis C. Roy, James D. Griffin, Marcia Belvin, Walter A. Blättler, John M. Lambert, and Jerome Ritz

The use of immunotoxins (IT) to selectively destroy acute myeloid leukemia (AML) cells in vivo or in vitro is complicated by both the antigenic similarity of AML cells to normal progenitor cells and the difficulty of producing a sufficiently toxic conjugate. The monoclonal antibody (MoAb) anti-MY9 is potentially ideal for selective recognition of AML cells because it reacts with an antigen (CD33) found on clonogenic AML cells from greater than 80% of cases and does not react with normal pluripotent stem cells. In this study, we describe an immunotoxin that is selectively active against CD33+ AML cells: Anti-MY9-blocked-Ricin (Anti-MY9-bR), comprised of anti-MY9 conjugated to a modified whole ricin that has its nonspecific binding eliminated by chemical blockage of the galactose binding domains of the B-chain. A limiting dilution assay was used to measure elimination of HL-60 leukemic cells from a 20-fold excess of normal bone marrow cells. Depletion of CD33+ HL-60 cells was found to be dependent on the concentration of Anti-MY9-bR and on the duration of incubation with IT at 37°C. More than 4 logs of these leukemic cells were specifically depleted following short exposure to high concentrations (10^{-8} mol/L) of Anti-MY9-bR. Incubation with much lower concentrations of Anti-MY9-bR (10^{-10} mol/L), as compatible with in vivo administration, resulted in 2 logs of depletion of HL-60 cells, but 48 to 72 hours of continuous exposure were required. Anti-MY9-bR was also shown to be toxic to primary AML cells, with depletion of greater than 2 logs of clonogenic cells following incubation with Anti-MY9-bR 10^{-8} mol/L at 37°C for 5 hours. Activity of Anti-MY9-bR could be blocked by unconjugated Anti-MY9 but not by galactose. As expected, Anti-MY9-bR was toxic to normal colony-forming unit granulocyte-monocyte (CFU-GM), which expresses CD33, in a concentration- and time-dependent manner, and also to burst-forming unit-erythroid and CFU-granulocyte, erythroid, monocyte, megakaryocyte, although to a lesser extent. When compared with anti-MY9 and complement (C'), Anti-MY9-bR could be used in conditions that provided more effective depletion of AML cells with substantially less depletion of normal CFU-GM. Therefore, Anti-MY9-bR may have clinical utility for in vitro purging of AML cells from autologous marrow when used at high IT concentrations for short incubation periods. Much lower concentrations of Anti-MY9-bR that can be maintained for longer periods may be useful for elimination of AML cells in vivo.

IMMUNOTOXINS (IT) are hybrid molecules consisting of a monoclonal antibody (MoAb) covalently linked to a toxin. IT have several theoretical advantages over conventional therapeutic agents including selectivity for tumor cells[1] and potential delivery of extremely potent toxins.[2,3] However, there have been several obstacles to the use of IT in leukemia therapy. First, it has been difficult to find MoAbs directed specifically against antigens found on clonogenic leukemia cells and absent from most normal hematopoietic precursor cells. Second, it has been difficult to find a highly potent toxin without significant nonspecific toxicity. Most single chain toxins, like pokeweed antiviral protein (PAP), saporin, and gelonin, have shown little toxicity against leukemic targets when linked to MoAbs.[4] Similar results were obtained with immunotoxins containing ricin A-chain alone.

In this study, we report the development of a novel IT that is composed of the MoAb anti-MY9 and a modified whole ricin toxin. The MoAb anti-MY9 (anti-CD33) is potentially ideal for targeting of acute myeloid leukemia (AML) cells because it reacts with clonogenic AML cells from greater than 80% of cases.[5] Previous studies have shown that CD33 antigen is present on normal colony-forming unit granulocyte-monocyte (CFU-GM), on a fraction of burst-forming unit-erythroid (BFU-E) and CFU-granulocyte, erythroid, monocyte, megakaryocyte (CFU-GEMM),[5,6] and absent from normal pluripotent stem cells.[7,8] One of the most powerful natural toxins, whole ricin, consists of two chains: an A-chain that inactivates 60S ribosomes and a B-chain that binds to galactose moieties found on the surface of all eukaryotic cells. We have devised a strategy to irreversibly block the binding sites of whole ricin, effectively blocking nonspecific binding to normal cells, while leaving both chains for increased intracellular toxicity.[9] This "blocked-Ricin" has been covalently linked to anti-MY9 forming an IT with increased cytotoxic activity.

We evaluated the capacity of Anti-MY9-blocked-Ricin (Anti-MY9-bR) to selectively eliminate AML cells under conditions compatible with both in vitro and in vivo use. We found that Anti-MY9-bR was highly toxic against AML cells when used under conditions simulating marrow purging (high concentrations of IT for short incubation periods) or in conditions simulating in vivo infusions (lower concentrations of IT for longer exposures). Compared with anti-MY9 and rabbit complement (C'), Anti-MY9-bR induced more depletion of leukemic cells and was less toxic to CFU-GM. These findings suggest that this IT may have clinical utility as both a purging agent for autologous bone marrow transplantation (BMT) and as a direct therapeutic agent.

From the Division of Tumor Immunology, Dana-Farber Cancer Institute; Department of Medicine, Harvard Medical School, Boston; and ImmunoGen Inc, Cambridge, MA.

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Address reprint requests to Denis C. Roy, MD, Department of Hematology-Immunology, Maisonneuve-Rosemont Hospital, 5415 L'Assomption Blvd, Montreal, Quebec, Canada H1T 2M4.

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MATERIALS AND METHODS

Bone Marrow (BM) Cells

After obtaining informed consent under protocols approved by the Human Subjects Protection Committee of the Dana-Farber Cancer Institute, BM was obtained from healthy volunteer donors. BM mononuclear cells (BMMC) were isolated by Ficoll-Hypaque density gradient centrifugation. BMMC received 40 Gy of irradiation at 11.1 Gy/min (^{137}Cs; Gamma Cell, Atomic Energy of Canada, Ottawa) before they were mixed with leukemia cells in purging experiments.

Leukemic Cell Line

The cell line HL-60 is a human promyelocytic leukemia cell line expressing MY9 (CD33). Cells were grown in RPMI 1640 medium (GIBCO Labs, Grand Island, NY) containing 10% heat-inactivated fetal bovine serum (FBS; Hyclone Lab, Logan, UT), 2% L-glutamine, 1% sodium pyruvate, and 1% penicillin and streptomycin.

Primary Leukemic Cells

Clonogenic AML cells were obtained from diagnostic specimens of 12 untreated patients originating from peripheral blood and, rarely, BM (two patients) samples. AML was diagnosed according to French-American-British (FAB) classification.[10] Mononuclear cells were prepared by Ficoll-Hypaque sedimentation and cryopreserved in 10% dimethyl sulfoxide (DMSO) in the vapor phase of liquid nitrogen. All leukemia samples contained greater than 75% blasts. The percentage of blasts expressing MY9 antigen varied from 60% to 91%.

MoAbs and Immunoconjugates

Both Anti-MY9-bR and Anti-B4-bR were supplied by Immunogen Inc (Cambridge, MA). These IT were prepared in several steps as described by Lambert et al.[9] First, a triantennary *N*-linked oligosaccharide from fetuin was modified to afford a reagent that contained a terminal residue of 6-*N*-methylamino-6-deoxy-D-galactose on one branch of the triantennary structure and terminal galactose residues on the other two branches. The ligand was activated with cyanuric chloride and the resulting dichloro-triazine derivative of the ligand reacted with ricin, forming a stable covalent linkage. Anti-MY9-bR was made by covalently linking a single molecule of this irreversibly blocked ricin to anti-MY9, an IgG2a MoAb with anti-CD33 specificity[5] developed at ImmunoGen. Anti-B4-bR (anti-CD19-bR) consisted of an anti-B4 IgG1 antibody with specificity against a 95-Kd glycoprotein absent from AML cells[11] that was linked covalently to blocked-Ricin. Each IT had less than 0.1% free ricin and was pyrogen-free. Stable linkage of the MoAb to the blocked-Ricin was maintained for a period of at least 6 months when kept at 4°C. Anti-J5 was an IgG2a anti-CD10 MoAb (Coulter Immunology, Hialeah, FL).

Treatment of Cells

Immunoconjugates. Suspensions of leukemic cells with a 20-fold excess of irradiated BMMC were treated at 1×10^7 cells/mL with IT. Treatments were performed in RPMI 1640 supplemented with 2.5% FBS for high concentrations of IT (as compatible with in vitro marrow purging) and with 2.5% human AB serum (HAB) for low concentrations of IT (as compatible with in vivo administration). IT concentrations varied from 2.5×10^{-8} mol/L to 5×10^{-13} mol/L and the incubation period at 37°C ranged from 15 minutes to 72 hours.

Complements. Suspensions of leukemic cells with a 20-fold excess of irradiated BMMC were incubated at 1×10^7 cells/mL with MoAb at saturating concentrations for 15 minutes at 4°C followed by incubation with C′ (3- to 4-week-old rabbit serum; Pel-Freeze Inc, Brown Deer, WI) at 37°C for 30 minutes. Treatment with antibody and C′ was repeated twice.

Limiting Dilution Assay

After treatment with either IT or antibody and C′, cells were washed three times and plated in a limiting dilution assay (LDA) as described previously.[12] Briefly, each treatment sample was serially diluted from 5×10^5 to 0.5 cells per 100 μL in RPMI 1640 supplemented with 10% FBS. From 24 to 48 aliquots of each dilution were plated in flat bottom microculture plates (Nunclon, Nunc, Denmark). Cells were fed every 4 days and incubated at 37°C for 14 to 18 days. Growth at each serial dilution was assessed in an "all-or-nothing" (positive or negative) fashion under an inverted phase microscope. Frequency of clonogenic cells within the test population was estimated using χ^2 minimization, which was shown to provide maximum accuracy and precision.[13] This LDA allowed detection of a maximum of 4.4 ± 0.3 logs depletion of HL-60 cells in a 20-fold excess of normal BMMC.

CFU-GM and Leukemic Blast Colony Assay

BMMC or primary AML cells were treated with either IT or MoAb and C′. CFU-GM and leukemic blast colonies (CFU-L) were assayed in semi-solid agar (Agar Noble; Difco Laboratories, Detroit, MI) by a modification of the method of Pike and Robinson.[14] Underlayers (0.5 mL) were composed of 0.5% agar in Iscoves modified Dulbecco's minimum essential medium (IMDM) with 20% FBS. As a source of colony-stimulating factor, 20% conditioned medium from the bladder carcinoma 5637 cell line was added to the underlayer. The overlayer (0.5 mL of 0.3% agar) contained 0.5 to 3.0×10^5 normal BMMC or AML cells. The cultures were set up in quadruplicate in 24-well plastic culture plates (Linbro; Flow Laboratories Inc, McLean, VA) and incubated at 37°C in 5% CO_2 and humidified air. After 7 and 14 days of culture, overlayers were removed, dried onto glass slides, and stained with Gill's hematoxylin (Fisher Scientific, Orangeburg, NY). CFU-GM colonies were considered as aggregates of greater than 40 cells, clusters as aggregates of 8 to 40 cells. All primary AML colonies stained positively for either specific or nonspecific esterase, or both. CFU-L colonies were considered as aggregates of greater than 20 blast cells.

BFU-E and CFU-GEMM Assay

Erythroid colonies were grown in IMDM containing 0.9% methylcellulose, 30% FBS, 0.9% bovine serum albumin (BSA), 2×10^{-4} mol/L 2-mercaptoethanol, 2 U/mL erythropoietin (Amgen Biologicals, Thousand Oaks, CA), and 10% Mo cell-conditioned medium.[15] Cells were plated at 10^5/mL and the large, multicentric, and late hemoglobinizing bursts scored as BFU-E at 14 days. In other replicate cultures, the addition of erythropoietin was delayed for 3 to 5 days, and CFU-GEMM were enumerated in these cultures after 14 days.[16]

RESULTS

Effect of Concentration and Length of Incubation With Anti-MY9-bR on Leukemic Cell Depletion Using a Limiting Dilution Assay

High Anti-MY9-bR concentrations. To determine the effectiveness of Anti-MY9-bR for depletion of leukemic

cells from a marrow graft, we used an in vitro model in which HL-60 cells (CD33+) were mixed with a 20-fold excess of normal irradiated BMMC. This mixture was treated with various concentrations of Anti-MY9-bR for 2 hours and plated in a limiting dilution assay. As controls, cells were treated with either anti-MY9 (CD33) antibody alone or Anti-B4-bR, an IT recognizing CD19, an antigen present on B cells but absent from HL-60 cells. Anti-MY9-bR depleted HL-60 cells in a concentration-dependent manner (Fig 1A). Treatment with 5×10^{-11} mol/L Anti-MY9-bR depleted less than 1 log of leukemic cells, while treatment with 10^{-8} mol/L Anti-MY9-bR eliminated 4.4 logs of HL-60 cells. Treatment with Anti-B4-bR was not significantly more toxic than media control, even at the highest concentration (10^{-8} mol/L). When Anti-MY9-bR 10^{-8} mol/L was incubated for 2 hours with Nalm-6 cells, a cell line not expressing CD33, no cytotoxicity was detected (data not shown).

The cytotoxic activity of Anti-MY9-bR was also found to be dependent on time of exposure (Fig 1B). Anti-MY9-bR (5×10^{-9} mol/L) destroyed 1 log of leukemia cells after 15 minutes, and up to 3.6 logs after 6 hours. Under these conditions, Anti-B4-bR was not toxic to HL-60 cells.

Low Anti-MY9-bR concentrations. To simulate conditions compatible with in vivo administration of Anti-MY9-bR, HL-60 cells were treated with lower concentrations of

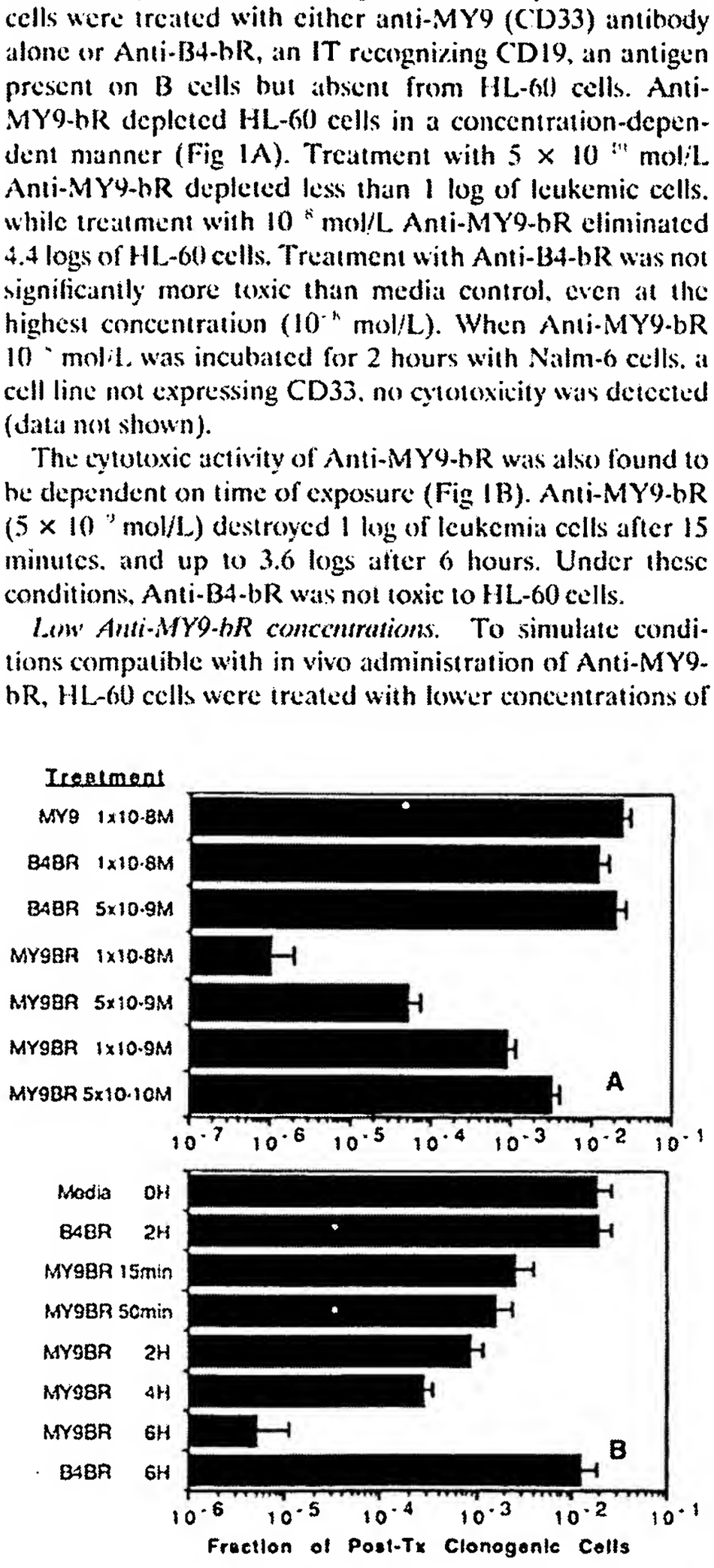

Fig 1. Effectiveness of Anti-MY9-bR for depletion of leukemia cells using limiting-dilution analysis. (A) HL-60 cells in a 20-fold excess of irradiated normal BMMC were incubated for 2 hours with unconjugated anti-MY9 (MY9), Anti-B4-bR (B4BR), and Anti-MY9-bR (MY9BR) at concentrations varying from 10^{-8} mol/L to 5×10^{-10} mol/L. (B) HL-60 + BMMC were incubated with Anti-MY9-bR 5×10^{-9} mol/L for up to 6 hours. The number of post-Tx clonogenic cells is expressed as a fraction (mean ± SE of two experiments) of the total number of cells plated (HL-60 + BMMC).

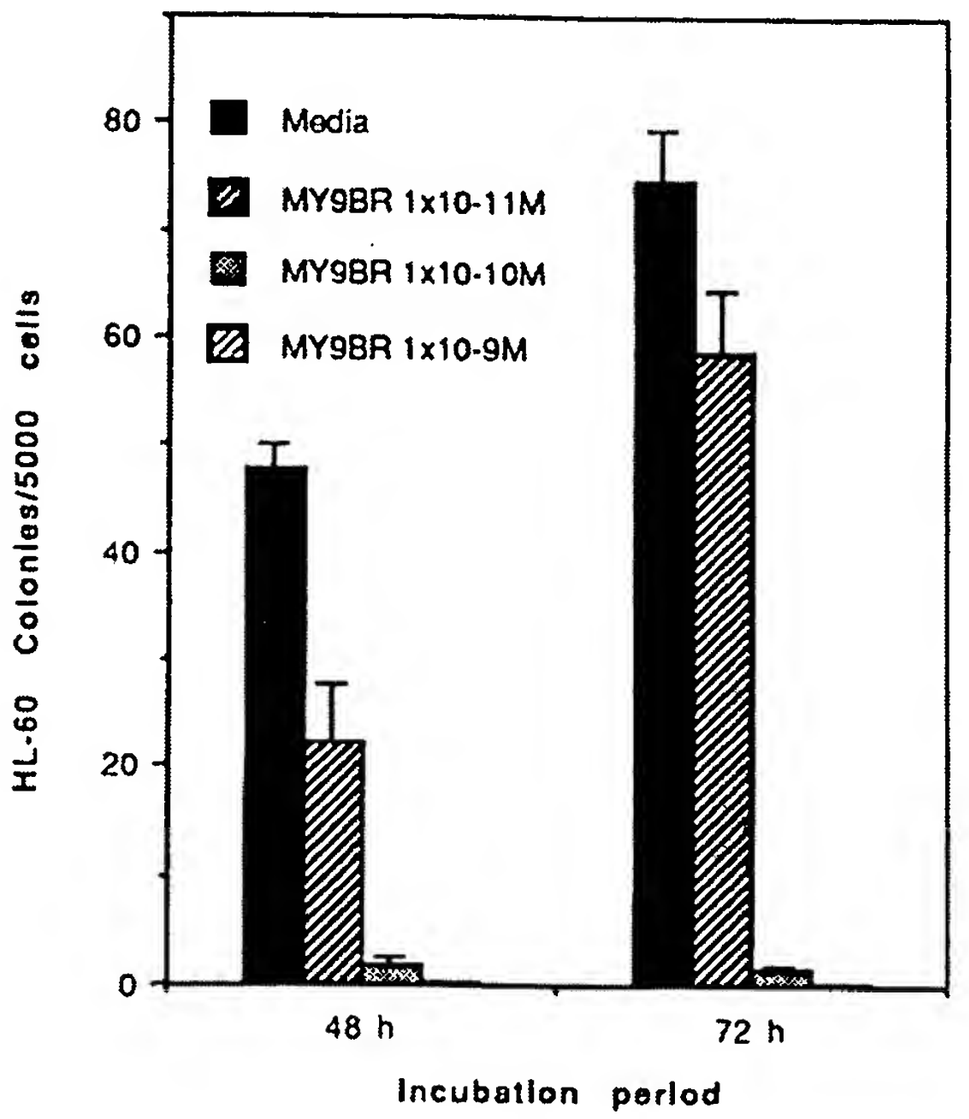

Fig 2. Effect of Anti-MY9-bR used at low concentrations for prolonged incubation periods. HL-60 cells were incubated for 48 and 72 hours with Anti-MY9-bR at concentrations of 10^{-9} mol/L to 10^{-11} mol/L and clonogenic growth (mean ± SE) evaluated in semisolid agar.

Anti-MY9-bR for more prolonged periods of time. As shown in Fig 2, incubation for 48 hours with Anti-MY9-bR at 10^{-10} mol/L, 10^{-10} mol/L, and 10^{-9} mol/L eliminated 0.33, 1.4, and 2.4 logs of clonogenic cells, respectively, as assayed in semisolid agar. Leukemic cells incubated in media for 72 hours grew more than cells incubated in media for 48 hours, but the extent of depletion nevertheless depended on the period of incubation with Anti-MY9-bR (Fig 2). Anti-MY9-bR at 10^{-10} mol/L for 72 hours destroyed 1.7 logs of these cells and at 10^{-9} mol/L induced depletion of at least 2.6 logs of clonogenic cells (the lower limit of detection of this assay).

Mechanism of Anti-MY9-bR Toxicity

To evaluate if the B-chain binding sites of blocked-ricin in Anti-MY9-bR were completely blocked, we used our LDA assay to measure cytotoxicity towards CD33+ target cells in the presence and absence of galactose (Fig 3A). Incubation of Anti-MY9-bR (5×10^{-9} mol/L) with galactose (0.1 mol/L) for 90 minutes depleted the same number (1.5 ± 0.1 log) of HL-60 cells in BMMC as incubation with Anti-MY9-bR alone. In contrast, after addition of a 100-fold excess of unconjugated anti-MY9 MoAb, depletion of HL-60 cells by Anti-MY9-bR decreased dramatically. Addition of both galactose and anti-MY9 to Anti-MY9-bR did not inhibit further the activity of Anti-MY9-bR, confirming specific targeting of leukemia cells through the MoAb moiety.

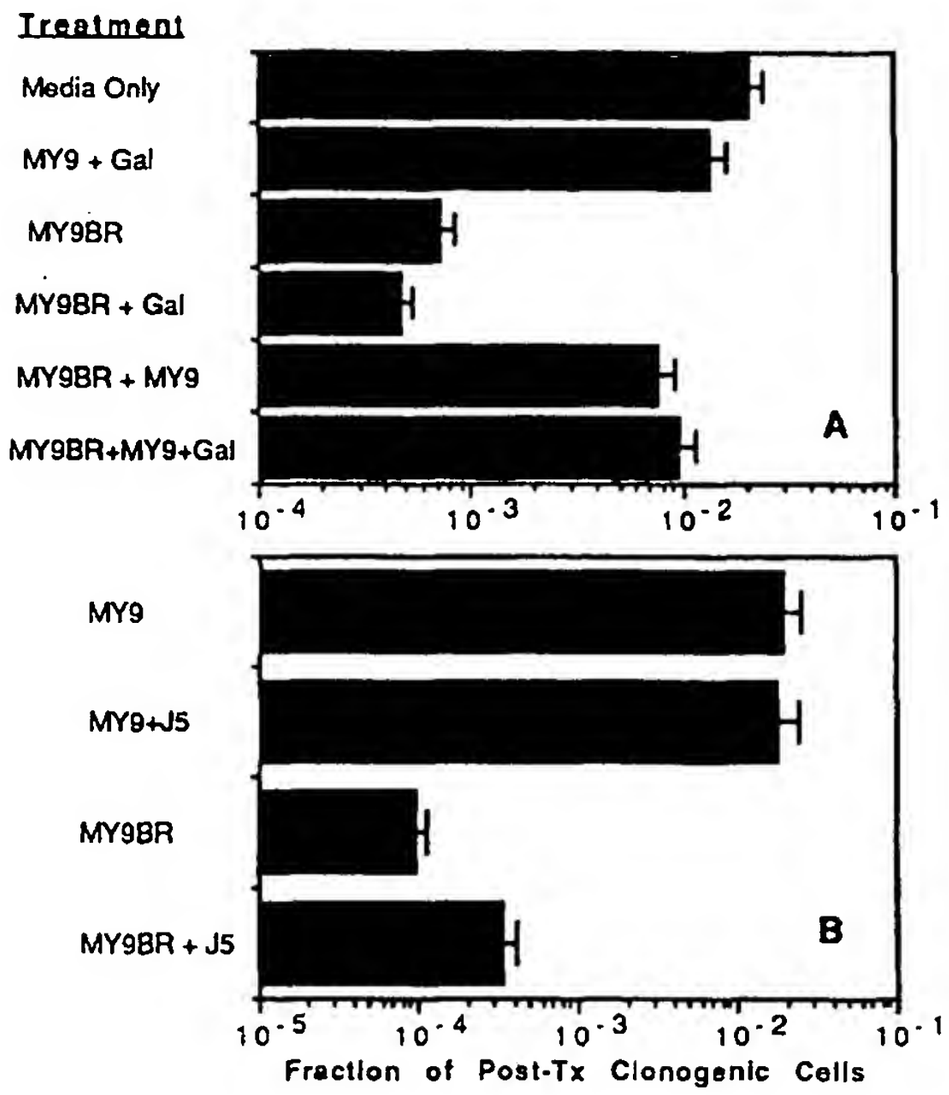

Fig 3. Specificity of the anti-leukemia activity of Anti-MY9-bR. HL-60 + BMMC were incubated with (A) Anti-MY9-bR 5×10^{-9} mol/L for 90 minutes in the presence of galactose 0.1 mol/L (Gal) and/or a 100-fold excess of anti-MY9 MoAb (CD33). The possibility of Fc binding was evaluated by incubating HL-60 + BMMC with (B) Anti-MY9-bR 5×10^{-9} mol/L for 2 hours with or without adding an MoAb of the same IgG2a isotype (J5).

Anti-MY9-bR could still have bound to targets through the Fc portion instead of the Fab portion of its anti-MY9 MoAb moiety. To examine this possibility, a 100-fold excess of an irrelevant MoAb of the same IgG2a isotype, anti-J5 (CD10), was added to Anti-MY9-bR. Anti-J5 does not react specifically with target cells but Fc binding does occur and competes with Fc binding of anti-MY9. Incubation of HL-60 cells alone with Anti-MY9-bR (5×10^{-9} mol/L for 2 hours) inhibited growth of 98.6% ± 0.1% (mean ± SE) of HL-60 cells as measured with the same LDA, while the addition of anti-J5 to Anti-MY9-bR caused 91.9% ± 1.1% inhibition of growth. This 6.7% difference in the elimination of HL-60 cells may be attributable to Fc binding. When HL-60 cells were treated in the presence of BM (Fig 3B), Anti-MY9-bR eliminated 99.5% ± 0.1% of leukemic cells, and after addition of anti-J5, 98.3% ± 0.2% of HL-60 cells was inhibited. Thus, the activity of Anti-MY9-bR decreased only slightly in the presence of anti-J5, especially in the presence of BM, suggesting that little Fc binding was occurring and that binding through the antigen-binding sites of anti-MY9 mediated killing by Anti-MY9-bR.

Effect of Cell Concentration on Leukemic Cell Depletion With Anti-MY9-bR

To determine the optimal conditions for in vitro treatment of BM with Anti-MY9-bR, we evaluated the effect of cell density on depletion of HL-60 cells in a 20-fold excess of BMMC. Five different concentrations of HL-60 + BMMC (ranging from 1×10^7 to 1×10^8 total cells/mL) were treated with either unconjugated anti-MY9 or Anti-MY9-bR at 5×10^{-9} mol/L for 2 hours at 37°C. For each cell concentration evaluated, the ratio of HL-60 cells to BM cells was constant. Over the range tested, concentration did not affect Anti-MY9-bR toxicity, which was 2.1 ± 0.2 logs.

Anti-MY9-bR Is Toxic to Primary Clonogeneic AML Cells

The cytotoxic activity of Anti-MY9-bR was subsequently measured against clonogenic AML cells obtained from 12 different patients. At high concentrations of Anti-MY9-bR (10^{-9} mol/L to 10^{-8} mol/L), elimination of AML cells was dose- (Fig 4A) and time-dependent (Fig 4B). Depletion of 94.7% of AML cells occurred after 2 hours of incubation with Anti-MY9-bR 10^{-8} mol/L. With this short incubation period, very low concentrations (2×10^{-10} mol/L) of Anti-MY9-bR were not toxic. Anti-B4-bR was nontoxic under identical conditions. Elimination of primary AML cells with lower concentrations of Anti-MY9-bR for longer incubation periods was also evaluated. At these lower concentrations (5×10^{-13} mol/L to 10^{-9} mol/L), increasing the incubation period with Anti-MY9-bR again showed elimination of AML cells in a dose- and time-dependent manner. Thus, 46% of AML cells were depleted after 24 hours of incubation with Anti-MY9-bR (5×10^{-11} mol/L) (data not shown) and prolonging the incubation to 48 hours increased depletion of primary AML cells to 78% (Fig 4C). Even concentrations of Anti-MY9-bR as low as 5×10^{-13} mol/L eliminated 28% of leukemic cells when the incubation period was extended to 48 hours.

Comparison of Anti-MY9-bR and Anti-MY9 Plus Rabbit Complement

The efficacy of in vitro treatment with Anti-MY9-bR IT used in conditions simulating autologous tumor cell purging was compared with that of treatment with anti-MY9 MoAb and baby rabbit complement. Three sequential treatments with anti-MY9 + C′ depleted 3.6 ± 0.3 logs of clonogenic HL-60 cells in a 20-fold excess of normal irradiated BMMC while anti-MY9 alone or complement alone did not cause significant killing. Treatment with Anti-MY9-bR 10^{-8} mol/L for 5 hours eliminated all detectable leukemic cells (LDA detection threshold = 4.4 ± 0.3 logs of HL-60 cells).

Effect of Anti-MY9-bR on Normal Hematopoietic Progenitor Cells

Because some normal hematopoietic progenitors express CD33 (MY9) antigen, we evaluated the toxicity of Anti-MY9-bR against CFU-GM, BFU-E, and CFU-GEMM. With a 2-hour incubation period, elimination of CFU-GM by Anti-MY9-bR (10^{-8} mol/L) was concentration-dependent (Fig 5A). Longer periods of exposure to Anti-MY9-bR (2.5×10^{-10} to 10^{-8} mol/L) resulted in increased toxicity (Fig 5B). Incubation of BMMC with 2.5×10^{-9} mol/L Anti-MY9-bR for 14 and 24 hours destroyed 76.3% and 89.3% of CFU-GM, respectively. Normal BM was also incubated with anti-MY9 or Anti-MY9-bR at the highest

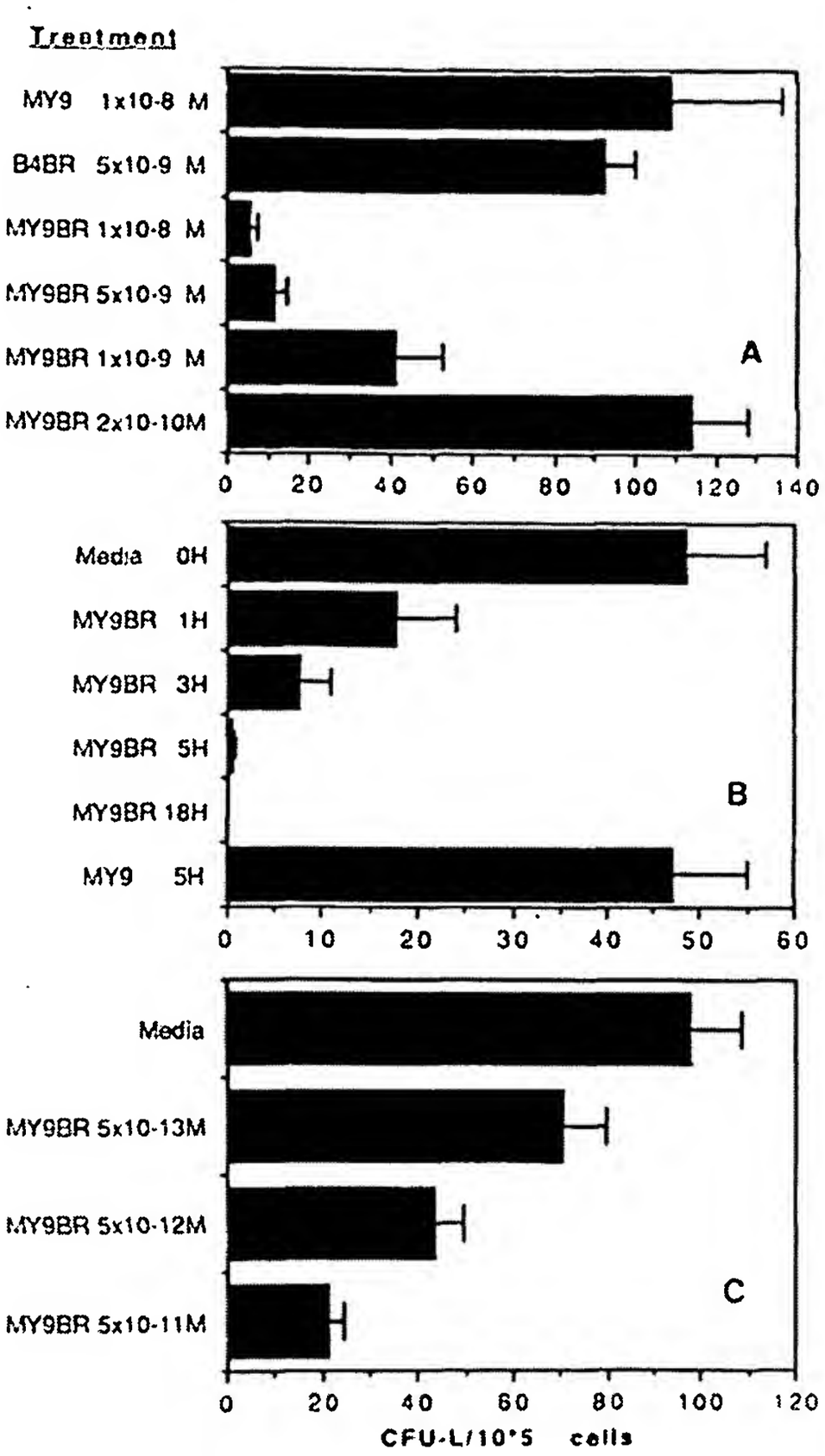

Fig 4. Activity of Anti-MY9-bR on primary AML cells. Clonogenic growth of primary AML cells after incubation with (A) high concentrations (10^{-8} mol/L to 2×10^{-10} mol/L) of unconjugated anti-MY9, Anti-B4-bR, or Anti-MY9-bR for 2 hours (mean ± SEM of AML cells from three patients), (B) unconjugated anti-MY9 or Anti-MY9-bR (10^{-8} mol/L) for incubation periods varying from 0 to 18 hours (mean ± SEM of AML cells from three patients), and (C) low concentrations of Anti-MY9-bR (5×10^{-11} to 5×10^{-13} mol/L) for 48 hours (mean ± SEM of AML cells from six patients).

concentration (10^{-7} mol/L) for periods up to 18 hours. Recovery of CFU-GM, BFU-E, and CFU-GEMM was identical after treatment with anti-MY9 MoAb or media alone. As shown in Table 1, increasing the incubation period with Anti-MY9-bR gradually decreased recovery of day 7 and 14 CFU-GM. After 5 hours of incubation with Anti-MY9-bR 10^{-8} mol/L, 70.7% and 77.3% of day 7 and 14 CFU-GM were depleted, respectively. After 18 hours, the same concentration of IT inhibited growth of more than 99% of CFU-GM.

The effect of different incubation periods with Anti-MY9-bR 10^{-7} mol/L on BFU-E and CFU-GEMM is also detailed in Table 1. Inhibition of BFU-E and CFU-GEMM clonogenic cells increased progressively with the length of the incubation period with Anti-MY9-bR. After 18 hours of incubation, Anti-MY9-bR inhibited growth of BFU-E by 46.1% and CFU-GEMM by 76.9%.

Comparison of Anti-MY9-bR Versus Anti-MY9 + C' Toxicity Against Normal Hematopoietic Progenitors

The number of normal hematopoietic progenitors remaining after treatment with unconjugated anti-MY9 and C' for three cycles was compared with that after treatment with

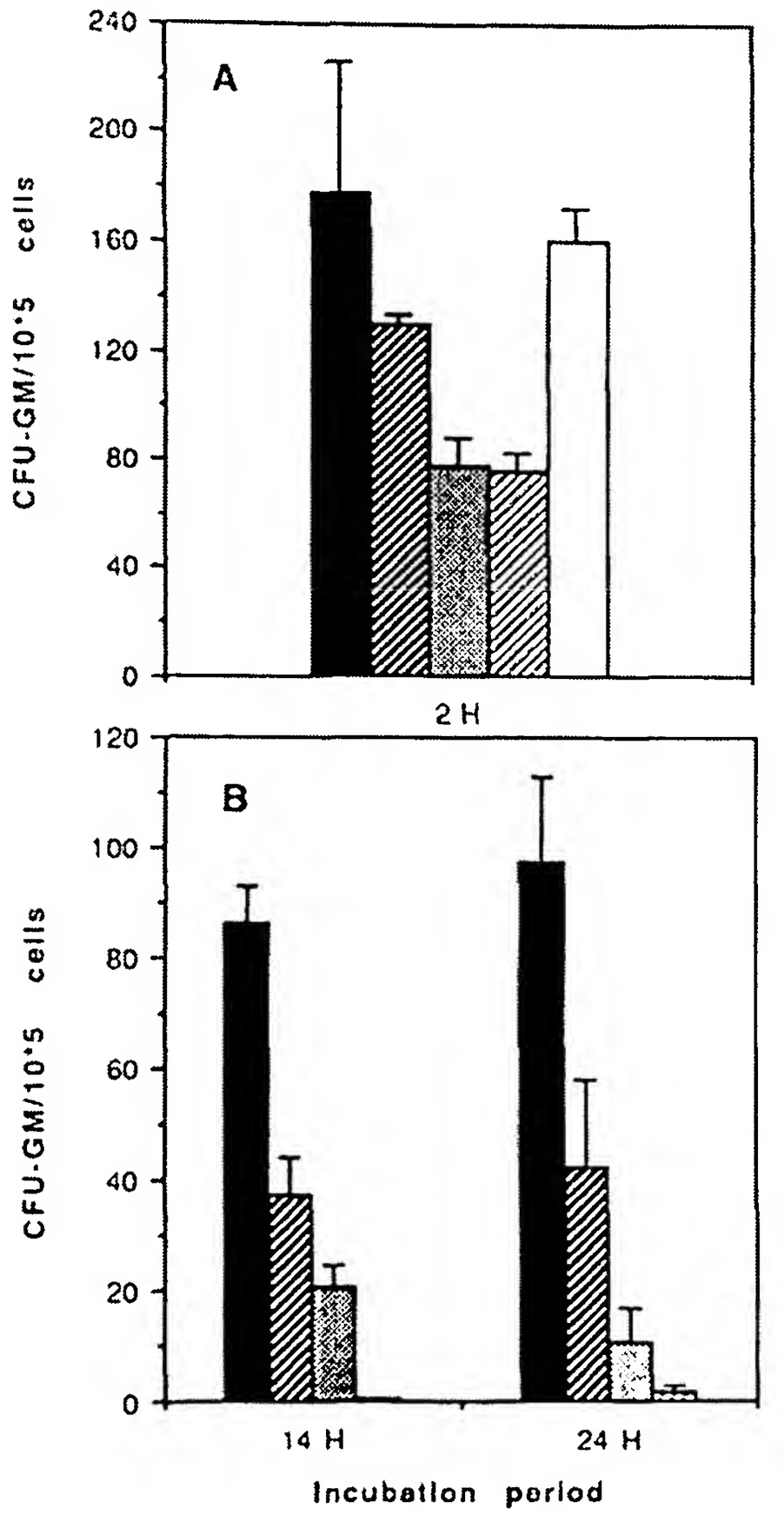

Fig 5. Toxicity of Anti-MY9-bR on CFU-GM progenitors. Normal BMMC were incubated for (A) 2 hours and (B) 14 and 24 hours with media alone, Anti-MY9-bR, or Anti-B4-bR at concentrations ranging from 10^{-8} mol/L to 2.5×10^{-10} mol/L and the number of CFU-GM colonies and clusters (mean ± SEM) scored after 14 days. Concentrations are as follows: (A) (■) Media. MY9BR: (□) 1×10^{-9}, (▨) 5×10^{-9}, (▧) 1×10^{-8} mol/L. ( ) B4BR 1×10^{-8} mol/L. (B) (■) Media. MY9BR: (□) 2.5×10^{-10}, (▨) 2.5×10^{-9}, (▧) 2.5×10^{-8} mol/L.

Table 1. Effect of Anti-MY9-bR Incubation Times on Hematopoietic Progenitors

Progenitor Cells	Control*	Anti-MY9-bR 1 h†	Anti-MY9-bR 3 h	Anti-MY9-bR 5 h	Anti-MY9-bR 18 h‡
CFU-GM	126.8 ± 33.4	79.8 ± 30.5	66.2 ± 17.7	37.2 ± 16.3	0.8 ± 0.5
Day 7	(0)	(37.1)	(47.8)	(70.7)	(99.1)
CFU-GM	69.7 ± 22.2	32.2 ± 13.9	23.2 ± 9.1	15.8 ± 6.6	0.3 ± 0.3
Day 14	(0)	(53.8)	(66.7)	(77.3)	(99.4)
BFU-E	32.6 ± 5.1	24.6 ± 3.8	19.5 ± 3.0	18.9 ± 2.7	6.9 ± 0.9
	(0)	(24.5)	(40.2)	(42.0)	(46.1)
CFU-GEMM	2.3 ± 0.4	2.2 ± 0.5	1.6 ± 0.4	1.3 ± 0.3	0.3 ± 0.2
	(0)	(4.3)	(30.4)	(43.5)	(76.9)

Mean ± SD (% inhibition of growth calculated as compared with control) of three experiments with each count in quadruplicate.

*Anti-MY9 unconjugated antibody (10^{-8} mol/L) incubated at 37°C for 5 hours.

†Anti-MY9-bR (10^{-8} mol/L) incubated at 37°C for all incubation periods.

‡(% inhibition of growth) for Anti-MY9-bR 18 hours is calculated as compared with BMMC cultured in anti-MY9 unconjugated MoAb for 18 hours.

Anti-MY9-bR 10^{-8} mol/L for 5 hours. As shown in Table 2, CFU-GM were decreased after both types of marrow treatments, but while anti-MY9 + C′ treatment eliminated all day 7 and 14 CFU-GM, Anti-MY9-bR preserved some CFU-GM ($P < .005$). Although there was large variability in the number of BFU-E and CFU-GEMM remaining in different patient samples, we found no difference in the inhibition of BFU-E and CFU-GEMM after treatment with either anti-MY9 + C′ or anti-MY9-bR.

Comparative Activities of Anti-MY9-bR Activity Against Various Targets

The overall effect of Anti-MY9-bR concentration on depletion of different MY9+ cell populations was evaluated in conditions simulating in vitro and in vivo administration. Results following incubation of HL-60, primary AML, and normal BM cells with Anti-MY9-bR at relatively high concentrations (5×10^{-10} mol/L to 10^{-8} mol/L) for short (2-hour) incubation periods are compiled in Fig 6A. HL-60 cells were more sensitive to Anti-MY9-bR than primary AML cells. Interestingly, primary AML cells were more sensitive to Anti-MY9-bR than CFU-GM. When the incubation period was prolonged to 24 hours, lower concentrations of Anti-MY9-bR were found to exhibit significant cytotoxicity. As shown in Fig 6B, HL-60 cells were more sensitive to Anti-MY9-bR than primary AML cells. In contrast to short incubations, primary AML cells and CFU-GM cells were similarly sensitive to long (24-hour) incubations with Anti-MY9-bR. For comparison, the same concentrations of Anti-MY9-bR caused little toxicity to CD33− Namalwa target cells.

DISCUSSION

We report here an IT that is highly toxic to AML cells in vitro. The first feature differentiating Anti-MY9-bR from other IT is its toxin moiety, which consists of both ricin A-chain and a modified B-chain that has its galactose binding sites blocked. Previous experiments with conjugates of anti-MY9 linked to purified ricin A-chain showed little cytotoxicity (J.D. Griffin, unpublished results), suggesting that the functions of the B-chain could not be entirely replaced by a MoAb. When free B-chains were added to single ricin A-chain IT, the cytotoxicity was markedly enhanced,[17] and it was suggested that the B-chain possibly facilitated transport of the IT into target cells.[18] In this study, whole "blocked" ricin conjugate displayed high levels of toxicity against CD33+ cells, providing support to the hypothesis that both chains, and possibly linkage of these chains, play an important role in generating cytotoxicity.

Several IT have been developed, mostly against T- and B-cell surface receptors,[2-4] but Anti-MY9-bR is one of the few reported IT with selective activity against myeloid cells[19] and the first IT against AML cells with potential for in vivo therapy. The specificity of Anti-MY9-bR for CD33+ cells was shown by demonstrating that galactose, the natural ligand of ricin, did not inhibit cytotoxicity, suggesting that unblocked ricin was not mediating the observed cytotoxicity. Further, Anti-MY9-bR was greater than 3 logs more toxic for CD33+ cells than CD33− cells and the cytotoxicity could be blocked by an excess of unconjugated anti-MY9 MoAb. Finally, addition of an isotype-specific MoAb did not inhibit Anti-MY9-bR from killing HL-60

Table 2. Inhibition of Normal Hematopoietic Progenitors by Anti-MY9 + C′ and Anti-MY9-bR

	CFU-GM D7	CFU-GM D14	BFU-E	CFU-GEMM
Anti-MY9 + C′*	99.0 ± 1.2	99.0 ± 1.5	39.6 ± 32.2	52.5 ± 46.2
Anti-MY9-bR†	77.8 ± 13.3‡	87.6 ± 8.0‡	33.2 ± 46.0	34.5 ± 59.2

Mean ± SD of % inhibition of colony formation of at least three separate experiments with each measurement performed in duplicate or quadruplicate.

*Treatment with anti-MY9 + C′ repeated for a total of three cycles.

†Treatment with Anti-MY9-bR (10^{-8} mol/L) incubated at 37°C for 5 hours.

‡Anti-MY9 + C′ versus Anti-MY9-bR: $P < .005$.

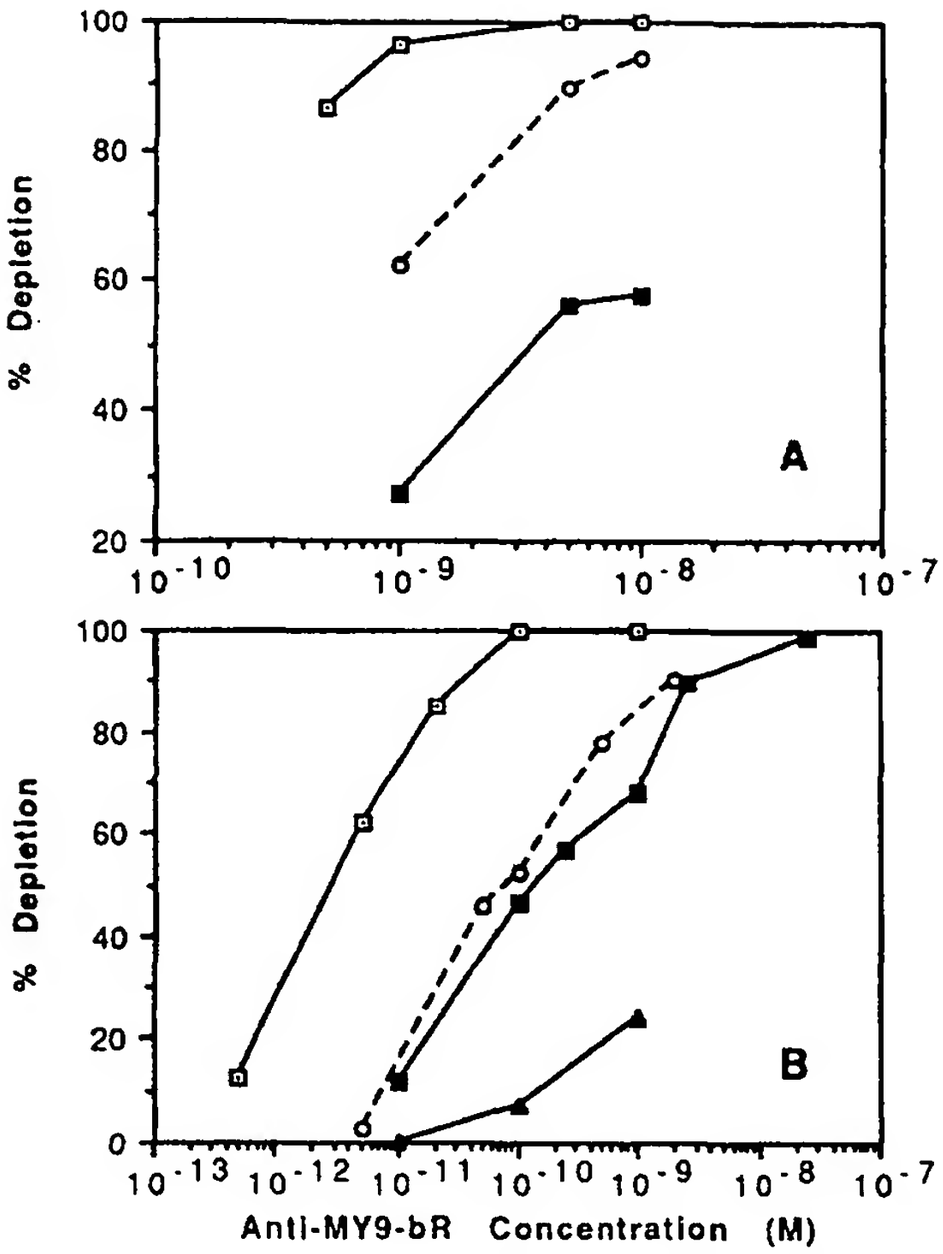

Fig 6. Comparative activities of Anti-MY9-bR on leukemia cells and normal hematopoietic progenitors. Percentage of depletion of HL-60 (—⊡—), primary AML (--○--), CFU-GM (—■—), and Namalwa cells (—△—) after (A) 2 hours and (B) 24 hours of incubation with Anti-MY9-bR at various concentrations.

cells, excluding the possibility that nonspecific Fc binding mediated the cytotoxicity of Anti-MY9-bR.

There is clinical evidence that patients with leukemia transplanted with autologous marrows in second or subsequent remission without purging of their marrow grafts do relatively poorly.[20,21] Thus, a number of studies have evaluated marrow purging to improve patient outcome. These studies have shown that autologous BMT was not only a feasible alternative for patients with ALL in second or subsequent complete remission who did not have an HLA-compatible donor,[22,23] but that the disease-free survival of patients transplanted with such purged marrows was not significantly different from that of patients treated by allogeneic BMT.[24] Similarly, purging methods, consisting mainly of 4-hydroperoxycyclophosphamide and MoAb + C′, were developed to eliminate residual AML cells before autologous BMT.[25,26] In this study, we showed that an IT, Anti-MY9-bR, was effective for purging of autologous BM, depleting more than 4.4 logs of leukemic cells. Interestingly, when Anti-MY9-bR was compared with anti-MY9 + C′, the IT could be used in conditions that eliminated more leukemia cells than anti-MY9 + C′. Compared with other purging methods, Anti-MY9-bR also provided the added benefit of not necessitating the complicated standardization procedures associated with complement use, or the sophisticated apparatus required for magnetic or laser purging methods. These observations suggest that Anti-MY9-bR is a potentially ideal agent for purging of AML cells.

The cytotoxic activity of Anti-MY9-bR against patient AML cells was evaluated using CFU-L as targets. More than 2 logs of these in vitro clonogenic AML cells, which are thought to represent the in vivo clonogenic AML cells,[6] were eliminated by this IT. In this study, the high levels of depletion of CFU-L exceeded the percentages (60% to 91%) of MY9-positive AML cells. This finding could be explained by the fact that some AML cells express few molecules of MY9 on their surface and escape detection by flow cytometry. In addition, it is also known that AML cells form a heterogeneous population of clonogenic and nonclonogenic cells. Previous studies have shown that these two populations of cells can be differentiated phenotypically.[6,27,28] Ia and MY9 antigens were found to be expressed on a significantly higher percentage of CFU-L than on the total AML cell population. In contrast, few CFU-L expressed Mo1 (CD11b) and Mo2 (CD14) and these antigens were found on a lower percentage of CFU-L than total AML cells.[28] These studies suggest that although AML cells have heteregeneous phenotypes, AML colony-forming cells are less heterogeneous and that the MY9 (CD33) antigen is expressed preferentially on the AML CFU-L.

One concern with using Anti-MY9-bR for purging is that it eliminates the majority of myeloid progenitors and could therefore prevent hematologic engraftment. At our institution, 11 patients have undergone autologous BMT with purging of the marrow graft using anti-MY9 + C′ treatment.[29] All patients engrafted, with a median time to absolute neutrophil count greater than 500/μL of 45 days after BMT (range 16 to 75). Time to white blood cell (WBC) engraftment was longer in these patients than in patients transplanted with autologous MoAb + C′ purged marrows for acute lymphoblastic leukemia[23] or lymphoma.[30] Although patients with AML had delayed engraftment, which correlated with total elimination of CFU-GM progenitors after anti-MY9 + C′ treatment, all these patients engrafted. Hematologic reconstitution thus probably originated from CFU-GEMM progenitors, which were only partially eliminated by such treatment, and from the hematopoietic stem cells that do not express CD33. The fact that all patients engrafted after anti-MY9 + C′ marrow treatment suggests that marrow treatment with Anti-MY9-bR, which targets the same MY9+ cells, will not prevent engraftment. In addition, Anti-MY9-bR, used in conditions for in vitro purging, depleted significantly less CFU-GM than anti-MY9 + C′. This finding suggests that patients receiving a marrow treated with Anti-MY9-bR might engraft more rapidly than after anti-MY9 + C′ treatmcnt of the marrow, decreasing risks for infections and improving patient prognosis.

Surprisingly, when we compared the effect of Anti-MY9-bR on CFU-L and CFU-GM, we found that, for short incubation periods, leukemia cells were more sensitive to Anti-MY9-bR than normal hematopoietic progenitors. This difference in sensitivity between primary AML cells and

CFU-GM is difficult to explain because both types of cells express high levels of CD33 antigen.[5,28] It is possible that normal and leukemic cells have different internalization rates of IT, different sensitivities to intracellular IT, or different intracellular metabolism of IT. Purging of BM grafts provides an opportunity to take advantage of these different sensitivities of normal and leukemic cells to short incubations of Anti-MY9-bR.

An important aspect of IT is their potential for in vivo use. Early trials in which MoAb only were given intravenously to patients with leukemia showed that antibodies were effectively delivered to leukemic cells in both peripheral blood and BM, yet the clearance of circulating cells was transient and BM blasts were not affected.[31] These transient responses were primarily due to the limited host mechanisms available to kill antibody-coated leukemia cells.[32] Linking a toxic molecule to the MoAb could solve many of the problems of low leukemic toxicity, but covalent conjugation of MoAb with toxins has often been complicated by loss of activity.[2,3] In this study, we showed that blockage of galactose binding sites and coupling with anti-MY9 was achieved while preserving the cytotoxic potential of Anti-MY9-bR.

In vivo administration of IT is also very difficult because of the large distribution volume of the patient, IT metabolism, and an environment at 37°C that all contribute to decrease IT serum concentrations. In addition, enhancers like lysosomotropic amines or carboxylic ionophores,[33,34] that can be used in vitro to potentiate the toxicity of IT, are only active at very high concentrations that cannot be achieved in vivo. Thus, IT with potential for in vivo administration must be capable of maximal efficacy at low serum concentrations. A phase I study evaluating Anti-B4-bR, a very similar IT, for in vivo administration is presently ongoing at our institution. In these patients, doses of IT ranging from 10 to 30 μg/kg/d administered by continuous infusion were minimally toxic and achieved stable serum levels of 1 to 4 × 10^{-10} mol/L of Anti-B4-bR.[35] In the present study, Anti-MY9-bR at 10^{-10} mol/L depleted 1.7 logs of HL-60 cells after only 72 hours of incubation and even concentrations as low as 5 × 10^{-13} mol/L were able to eliminate 55% of primary AML cells after 48 hours without addition of enhancers. If we can extrapolate from these observations, Anti-MY9-bR should be a most active agent for in vivo treatment of patients with AML. A potential problem with prolonged infusions of Anti-MY9-bR is the depletion of MY9+ progenitors. In similar in vitro conditions, we found that CFU-GM were as sensitive to Anti-MY9-bR as AML cells. Thus, we would expect that doses of Anti-MY9-bR active against AML cells in vivo will also cause neutropenia. The fact that MY9 is absent from the surface of the hematopoietic stem cell combined with our previous experience with anti-MY9 + C′ purging of marrow grafts suggest that this toxicity will only be transient.

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A fusion DNA vaccine that targets antigen-presenting cells increases protection from viral challenge

Georgia Deliyannis*†, Jefferey S. Boyle†‡§, Jamie L. Brady‡, Lorena E. Brown*, and Andrew M. Lew‡¶

*Cooperative Research Center for Vaccine Technology, Department of Microbiology and Immunology, University of Melbourne, Melbourne 3052, Australia; †Walter and Eliza Hall Institute of Medical Research, Parkville 3050, Australia; and ‡CSL Limited, Parkville, Victoria 3052, Australia

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Improving the immunological potency, particularly the Ab response, is a serious hurdle for the protective efficacy and hence broad application of DNA vaccines. We examined the immunogenicity and protective efficacy of a hemagglutinin-based influenza DNA vaccine that was targeted to antigen-presenting cells (APCs) by fusion to CTLA4. The targeted vaccine was shown to induce an accelerated and increased Ab response (as compared with those receiving the nontargeted control) that was predominated by IgG1 and recognized conformationally dependent viral epitopes. Moreover, mice receiving the APC-targeted DNA vaccine had significantly reduced viral titers (100-fold) after a nonlethal virus challenge. The increased protective efficacy was most likely because of increased Ab responses, as cytotoxic T lymphocyte responses were not enhanced. Targeting was demonstrated by direct binding studies of CTLA4 fusion proteins to the cognate ligand (B7; expressed on APCs *in vivo*). In addition, a targeted protein was detected at 4-fold higher levels in draining lymph nodes within 2–24 h of administration. Therefore, this study demonstrates that targeting DNA-encoded antigen to APCs results in enhanced immunity and strongly suggests that this approach may be useful in improving the protective efficacy of DNA vaccines.

Immunization with DNA vaccines encoding antigen (Ag) has been used to induce both cellular and humoral immune responses and holds enormous potential for developing vaccines to a variety of pathogens. Recent reports of the first human clinical trials have shown that DNA vaccines were well tolerated, and the responses [especially cytotoxic T lymphocytes (CTLs)] were generally encouraging (1–3). However, there are still some questions concerning the potency of DNA vaccines, particularly with regard to Ab responses.

There is a low level of protein expression after DNA immunization (e.g., nanograms produced). We sought to enhance the effectiveness of this dose by delivering what small amount of Ag is made to the cells relevant to immune induction [i.e., Ag-presenting cells (APCs)]. We showed that increased Ab and T cell responses to a model Ag [human IgG (hIgG)] could be achieved by fusion of the Ag to CTLA4, which directs it to APCs through binding to the surface receptors B7-1 and B7-2 (4). Targeting APCs through the CD11c receptor has also been shown recently to enhance Ab responses (5). In this study, we sought to assess the efficacy of our targeting strategy in a viral challenge system. A plasmid expressing the influenza hemagglutinin (HA) molecule fused with CTLA4 was constructed and examined for its ability to enhance the immune response after DNA immunization and to reduce the amount of virus present in the lungs of mice after a nonlethal influenza challenge.

Materials and Methods

Plasmids. The backbone of all the plasmids used for immunization was the same, namely pCI (Promega), which contains a human cytomegalovirus promoter plus intron. Influenza A/Puerto Rico/8/34 H1 (PR8) HA was fused to hIg or CTLA4-hIg [the nontargeted and targeted molecules, respectively, described by Boyle *et al.* (4)], whereby hIg has the leader sequence of CD5 to afford secretion and the hinge, CH2 and CH3 regions of hIgG1 and CTLA4 is the ectodomain of CTLA4. This fusion was done by creating a gly-gly-gly-gly-thr spacer at the 3′ *Nsi*I site of hIg and adding the *Pvu*II-*Bst*YI fragment of HA (83–1609; GenBank accession no. J02143) 3′ of that spacer. This HA fragment represents the mature HA without the signal peptide and without the transmembrane domain. CTLA4-hIg-S was the control plasmid whereby HA was replaced by an irrelevant peptide (SIINFEKL) derived from chicken ovalbumin. HA plasmid encodes signal peptide and ectodomains of HA up to the same *Bst*YI site above. The plasmids used for immunization are represented in Fig. 1*d*. DNA was prepared by the polyethylene glycol/Triton X-114 method (6), so that there was <0.05 ng of endotoxin per mg of DNA.

Virus. Influenza virus A/PR8 (H1N1) was grown in the allantoic cavity of 10-day embryonated hens' eggs for 2 days at 35°C. The allantoic fluid was collected, clarified by centrifugation (2,000 × *g*, 15 min, 4°C), stored at −70°C, and used as a source of infectious virus for challenge of mice. Purified virus used in ELISAs and β-propiolactone-inactivated and sodium taurodeoxycholate-disrupted purified virus (split virus) for immunization of mice were obtained from CSL (Parkville, Victoria, Australia).

mAbs. mAbs E2.6 and C4.2 are directed against the HA of A/PR8 virus and recognize conformation-dependent and conformation-independent epitopes on the viral HA, respectively. mAb 165 binds to carbohydrate on glycosylated egg-grown influenza viruses and therefore also recognizes a conformation-independent epitope.

ELISA. The ELISA was performed as described (7) by using 96-well polyvinyl microtiter trays; each well was coated with 50 μl of a solution containing either purified intact virus (1,000 hemagglutinating units/ml) or split virus (5 μg/ml) per well. In ELISAs determining the levels of Abs recognizing denatured viral HA, the split PR8 virus was reduced and alkylated before coating. Anti-HA IgG subclass titers were determined by the method described (8). Briefly, bound Ab was detected after incubation with peroxidase-conjugated anti-mouse IgG1, IgG2a, or IgG2b Abs (Southern Biotechnology Associates) diluted in blocking buffer (5% skim milk powder in PBS) overnight at 4°C. Ab titers are expressed as the reciprocal of the Ab dilution giving an absorbance of 0.2 units.

Abbreviations: Ag, antigen; CTL, cytotoxic T lymphocyte; APC, antigen-presenting cell; hIgG, human IgG; PR8, influenza A/Puerto Rico/8/34 H1; HA, hemagglutinin.

†G.D. and J.S.B. contributed equally to this work.

¶To whom reprint requests should be addressed. E-mail: lew@wehi.edu.au.

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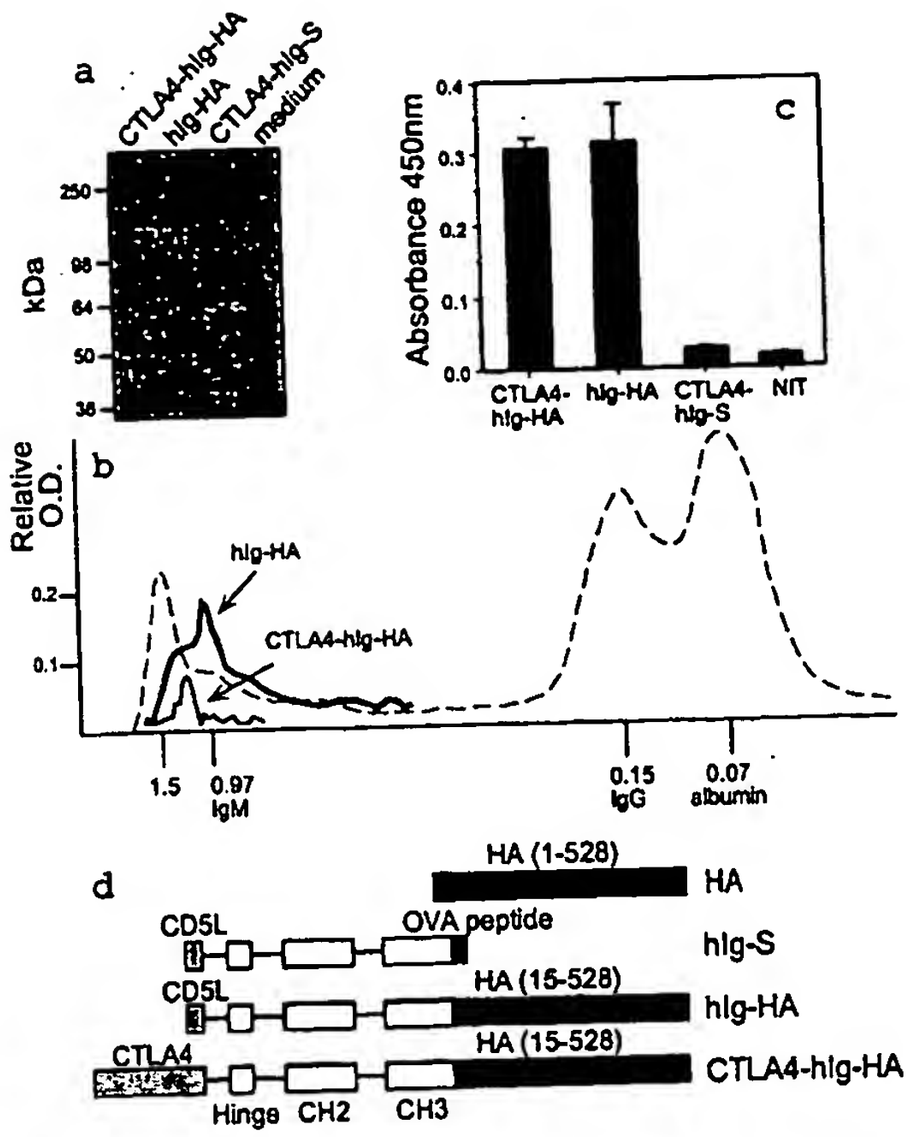

Fig. 1. Fusion molecules containing Ig-HA were secreted and dimerized. (*a*) Western blotting, after SDS/PAGE of proteins of transfected cells run under reducing conditions, by using peroxidase-conjugated goat anti-hIg and chemiluminescence. (*b*) Size chromatography of CTLA4-hIg-HA (thin line) and hIg-HA (thick line) from transfectant supernatants indicates that their M_r is 1.2 million and 1.0 million, respectively. Supernatants were concentrated and run at 24 ml/h on a Sephacryl S300 column (2.4 × 100 cm). The fractions were analyzed by capture ELISA for the presence of the fusion proteins. Only the positive fractions are shown. Blue dextran and sheep serum were run as size markers (shown as dashed line); numbers indicate the size in millions. (*c*) Capture ELISA of culture supernatants by using mAb E2.6 (which recognizes a conformationally dependent epitope) as capture Ab and peroxidase-conjugated goat anti-hIg crossadsorbed against mouse Ig as detecting Ab. (*d*) DNA constructs used for immunizations. All constructs were made on a pCI (Promega) backbone. Introns are indicated as lines connecting the exons of hIgG1 (hinge, CH2, and CH3) used. The amino acids of HA included are indicated in parentheses.

Cytotoxic T-Cell Assay. CTL assays were performed as described (9) by using P815 mastocytoma cells as targets.

Transfection, Western Blotting, Capture ELISA, and Molecular Sieving. NIT cells (an insulinoma cell line) were electroporated with 20 μg of plasmid and cultured for 3 days; the protein products were analyzed by capture ELISA or by Western blotting. Capture ELISA used the PR8 HA-specific mAb E2.6 as the capture Ab on the microtiter tray. Culture supernatants were added to the tray wells, and bound Ag was detected with a peroxidase-conjugated goat anti-hIgG crossadsorbed against mouse Ig (Southern Biotechnology Associates). For Western blotting of proteins after SDS/PAGE with or without 2-mercaptoethanol, bands were detected with the peroxidase-conjugated anti-hIg followed by chemiluminescence. The relative size of the proteins was estimated by running concentrated supernatants on a Sephacryl S300 column (2.4 × 100 cm) at 24 ml/h. Fractions were collected and analyzed by capture ELISA for the presence of fusion proteins.

B7-Binding Assay. Supernatants from Chinese hamster ovary cells transfected with the DNA expressing the CTLA4-hIg-HA, hIg-HA, or CTLA4-hIg-S plasmids were added to NIT cells expressing membrane-bound B7-1 or untransfected controls and bound protein detected with FITC-conjugated anti-hIg secondary Ab.

Immunization. Groups of 10 female 6- to 8-wk-old BALB/c mice, bred in the animal facility of the Department of Microbiology and Immunology (University of Melbourne), were immunized i.m. in both quadriceps under ketamine/xylazine anesthesia with 100 μg of plasmid DNA in saline. As a positive control, mice were immunized s.c. in the scruff of the neck with split virus in PBS.

Intranasal Challenge of Mice, Preparation of Lung Extracts, and Assay for Infectious Virus. Penthrane-anesthetized mice were challenged with 50 plaque-forming units of infectious virus by the intranasal route; each mouse received 50 μl of virus in the form of allantoic fluid diluted in PBS. At 5 days after challenge, mice were killed by cervical dislocation; lungs were removed and homogenized, and the supernatants were stored at −70°C before assay for infectious virus. Virus titers were determined by plaque assay on monolayers of Madin–Darby canine kidney cells as described (10).

Statistical Analysis. Data were analyzed by using the nonparametric Mann–Whitney *U* test, which compares two sets of unpaired samples. The null hypothesis is that the two population medians are equal, and the resultant *P* value for particular comparisons is given.

Results and Discussion

Expression of HA Constructs in Mammalian Cells. Mammalian expression plasmids were constructed to express HA under the control of the cytomegalovirus promoter. A targeted secreted fusion vaccine (CTLA4-hIg-HA) was made to encode CTLA4 at the N terminus for targeting, followed by the Fc of hIgG1 to promote dimerization for enhanced binding to B7 (11) and influenza HA at the C terminus. As controls, plasmid encoding a fusion molecule lacking the targeting ligand (hIg-HA) and another in which the HA was replaced with an irrelevant Ag sequence (CTLA4-hIg-S) were likewise made. Previously, we had shown that increased responses were achieved only when the Ag was fused with CTLA4 (4) and when a dimerization moiety (namely the Fc of IgG) was also present. The plasmids were first used to transfect cells *in vitro*. Western blotting confirmed the expected sizes of the reduced protein products to be 125 kDa and 145 kDa for hIg-HA and CTLA4-hIg-HA, respectively (Fig. 1*a*). Sephacryl chromatography revealed that the native structures were 1.0 and 1.2 million in size (Fig. 1*b*). These sizes are consistent with higher ordered structures (e.g., tetramers of dimers: the hIg acting as a dimerization moiety) (4). HA normally forms trimers, but higher-order structures may be formed from nonnative disulfide bonding or may be predisposed by the juxtaposition of HA by the hIg dimerization. In the context of this study, what is important is that both targeted and nontargeted molecules oligomerize similarly (the size ratio between subunit and native structure was similar for both), and thus differential oligomerization is not the reason for the differential Ab responses shown below. Furthermore, the protein levels after transfection were comparable between hIg-HA and CTLA4-hIg-HA. Secreted proteins in culture supernatants were also examined by capture ELISA by using an HA-specific mAb, and bound Ag was detected with peroxidase-conjugated Ab to hIgG. As expected, Ag was detected only in cultures transfected with plasmids encoding both HA and hIg sequences (Fig. 1*c*). Although the antigenic integrity of HA relies on the proper folding of the globular head domain

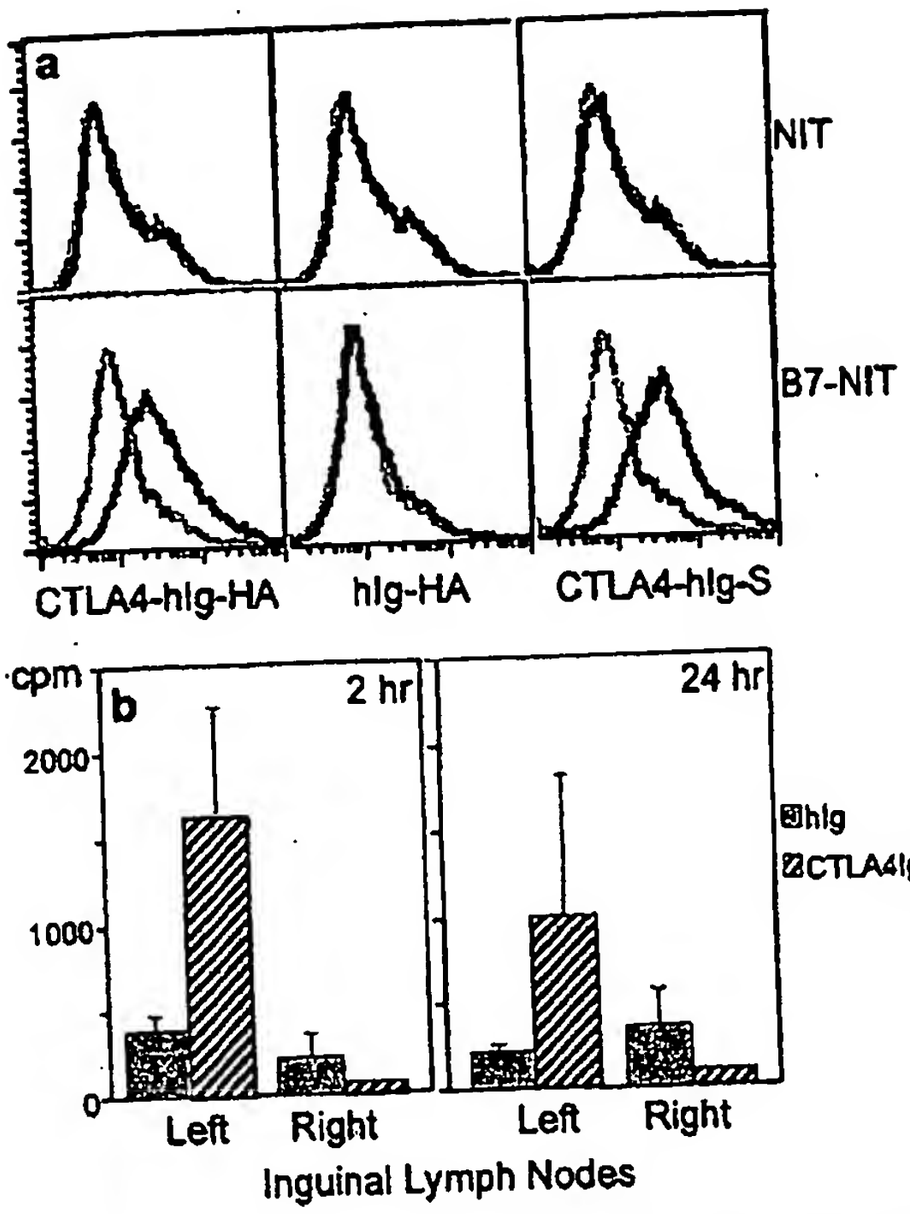

Fig. 2. Fusion proteins bind B7 and accumulate in the draining lymph node. (*a*) Flow cytometric analysis shows that CTLA4-hIg-HA binds to B7. NIT cells expressing membrane-bound B7-1 or untransfected cells (B7-NIT and NIT, respectively) were incubated with supernatants from Chinese hamster ovary cells transfected with CTLA4-hIg-HA, hIg-HA, or CTLA4-hIg-S (black line). The gray line represents the NIT or B7-NIT cells incubated with untransfected Chinese hamster ovary cell supernatants. FITC-conjugated sheep anti-hIgG was used as the secondary Ab. (*b*) Enhanced localization of CTLA4-hIg in draining lymph nodes. BALB/c mice were injected in the left quadriceps with radioiodinated CTLA4-hIg or hIg (total cpm was 3 million; total protein was 5 μg). Lymph nodes were harvested at 2 h and 24 h after immunization. The mean and SEM of five mice per group are shown.

(12), the mAb E2.6 used in the capture ELISA was a neutralizing Ab that recognizes a conformation-dependent epitope. This suggests that some conformational integrity has been maintained within the HA expressed from the hIg-HA and CTLA4-hIg-HA fusion vaccines. This was subsequently confirmed by the finding that the Ab responses obtained were largely against native HA (see below).

Targeting by the CTLA4 Ligand to Draining Lymph Nodes. The binding of the fusion vaccine was confirmed by flow cytometric analysis, and significant binding was detected only with the CTLA4-Ig fusion proteins (Fig. 2*a*). Our hypothesis is that the effectiveness of this ligand targeting is mainly because of physical delivery to the anatomical sites of immune induction, either directly or through association with migrating dendritic cells that bear the B7 molecule. Direct injection of DNA vaccines into the lymph nodes (13) or spleen (14) elicits potent immune responses, and we have shown that spleen injections are, in fact, superior to i.m. and intradermal injections (14). The alternative possibility, that the targeting molecule is actually providing signals to the APC, is less likely considering L-selectin can also be used as a targeting moiety to enhance the immune response to certain Ags (4); it is difficult to envisage how any signaling to high endothelial venule cells would lead to enhancement of a specific Ab response. Coinjection of L-selectin-hIg or CTLA4-hIg with ovalbumin does not enhance any responses to ovalbumin (4); therefore, the effect cannot be a nonspecific effect on homing or migration. In Fig. 2*b*, we also confirm that the amount of protein fused to CTLA4 found in draining (ipsilateral) lymph nodes is 4-fold greater than that of nontargeted protein at 2 h or 24 h postinjection ($P < 0.05$; compare with the fact that no difference was found for contralateral nodes). This preferential accumulation of the targeted protein is consistent with the hypothesis that physical delivery would seem to be a major, if not the only, mechanism for immune enhancement.

Enhancement of Ab Responses to DNA Immunization by Targeting. Groups of BALB/c mice ($n = 9$ or 10) were immunized on days 0 and 14 with 100 μg of the indicated DNA constructs in each quadriceps. As a positive vaccine control, an inactivated detergent-disrupted virus (split virus) that was produced in a manner similar to a current inactivated human influenza vaccine was injected. Sera were obtained at 2 and 4 weeks after initial immunization and assayed for antiviral Ab by ELISA. At 4 weeks, mice immunized with CTLA4-hIg-HA had produced the strongest Ab responses; these responses were significantly higher ($P < 0.001$) than those for the hIg-HA immunized controls (Fig. 3*a*). The Ab levels induced by CTLA4-hIg-HA immunization were comparable with those induced by the split virus control ($P = 0.57$; Fig. 3*a*). Mice receiving the DNA vaccine negative control (CTLA4-hIg-S) had no detectable antiviral Ab, whereas those that received the HA DNA mounted a low but detectable antiviral response (Fig. 3*a*). Interestingly, the 2- and 4-week responses to the DNA vaccines were similar, whereas the Ab levels in mice given split virus protein rose 4-fold after the second immunization (data not shown). This suggested that there was no requirement for, or benefit from, giving a second immunization of DNA (at 2 weeks) as was observed with split virus. Titers at 8 weeks were not significantly elevated from those at 2 or 4 weeks (data not shown); this is consistent with the observation that APC targeting speeds up the immune response for DNA vaccines (4, 5). To determine how much Ab elicited by the fusion DNA vaccines recognized the intact HA molecule, we compared the binding of Ab to native and denatured (reduced and alkylated) Ag (Fig. 3*b*). As controls, we used mAbs that recognized conformation-dependent and -independent epitopes. The relative binding for both hIg-HA and CTLA4-hIg-HA showed that most of the Ab elicited was against native epitopes, and that the relative binding was similar for both groups, suggesting that there was no difference in the way HA was folded between the two similarly fused proteins.

To examine further the form of the immune response, groups of five BALB/c mice were immunized with 100 μg of DNA; next, the relative HA-specific IgG subclass profile and, in a separate experiment, the CTL response were determined. All IgG subclasses tested (IgG1, IgG2a, and IgG2b) were elevated in the groups receiving the targeted vaccine, with the increase in IgG1 being the most substantial (Fig. 2*c*). There was no difference in the magnitude of the CTL response induced by the targeted and nontargeted vaccine (Fig. 2*d*). There have been several reports examining the mechanism of CTL induction after i.m. DNA immunization that indicate a role for crosspresentation of Ag through a bone marrow-derived APC (15, 16). As our targeting strategy may not affect crosspresentation, it is perhaps not surprising that CTLA4 targeting neither enhanced nor decreased CTL responses to influenza HA (Fig. 2*d*) and ovalbumin (data not shown).

Improved Protective Efficacy of the Targeted vs. Nontargeted DNA Vaccine. To determine the functional relevance of the immunity generated with the targeted vaccine, BALB/c mice were immu-

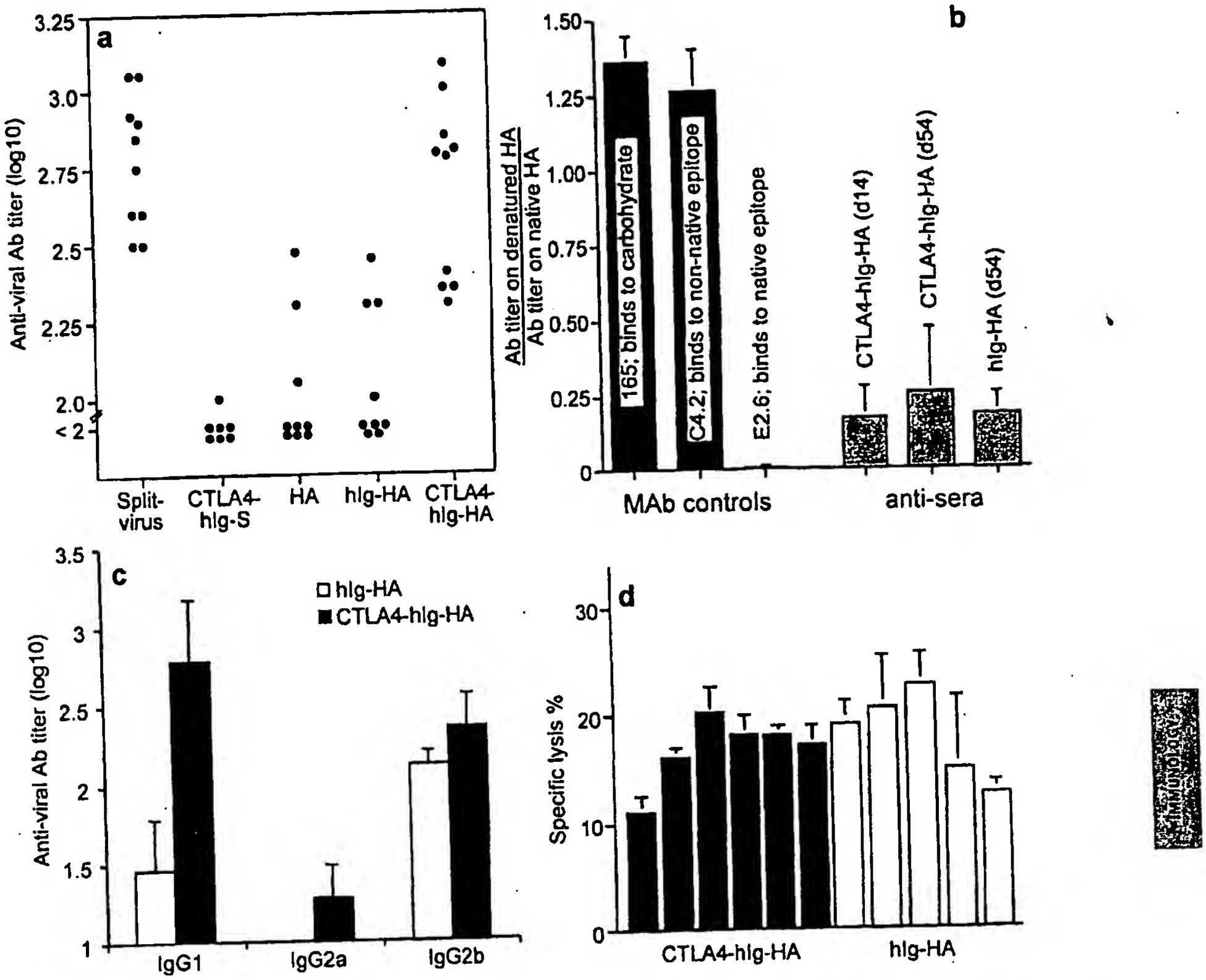

Fig. 3. Immune responses to the DNA vaccines. (*a*) Individual serum antiviral Ab titers after targeted DNA immunizations were greater than after nontargeted DNA immunization. Mice were immunized with 100 μg of plasmid DNA per leg in both quadriceps on days 0 and 14 and bled 2 weeks later or immunized with split virus as a control. (*b*) Antiviral Abs induced by CTLA4-hIg-HA and hIg-HA immunization recognize conformation-dependent epitopes on HA. Ab levels of mice at 14 and 54 days after priming were assayed by ELISA for binding to native split virus and denatured (reduced and alkylated) virus. For each mouse, the ratio of the titers of serum Ab recognizing denatured HA: native HA was calculated, and the mean and SD for each group were determined. As a control for relative coating levels and as an indicator of denaturation of PR8 HA, binding of mAbs 165 (recognizing a carbohydrate determinant), C4.2 (recognizing a conformation-independent determinant), and E2.6 (recognizing a conformation-dependent determinant) to native vs. denatured virus was also examined. It should be noted that the Ab levels of the untargeted DNA vaccine group on day 14 were too low for these relative binding data to be valid; therefore, these data are not shown. For the same reason, the data on day 54 for the untargeted group represent only three of 10 mice (the three highest responders). (*c*) IgG subclass responses. Groups of five female BALB/c mice were immunized with 100 μg of plasmid DNA; the IgG subclass titer was determined as the highest dilution to reach an OD of 0.2. The mean ± SEM for each group at 4 weeks is shown. (*d*) Targeted and nontargeted DNA vaccines elicit similar levels of virus-specific CTL activity. Mice were immunized I.m. with a single dose of 100 μg of plasmid DNA; after 4 weeks, individual spleens were taken, and single-cell suspensions were prepared. Cells were cultured for 5 days with virus-infected autologous spleen cells and subsequently tested in a ^{51}Cr release assay for their ability to lyse virus-infected and uninfected P815 target cells. Each bar represents the cytolytic response of an individual mouse at an effector-to-target ratio of 100:1. The mean of triplicate cultures is shown, and background lysis measured on uninfected targets has been subtracted.

nized with 100 μg of DNA and challenged 9 weeks later with infectious virus. The lung viral titers at 5 days after challenge were determined by plaque assay (Fig. 4). By comparing the geometric mean or the median titers in the control DNA vaccine-immunized group (hIg-HA), the viral load for the group that was immunized with CTLA4-hIg-HA was up to 100-fold lower ($P = 0.0004$) (Fig. 4). Given that the CTL responses were equivalent, we argue that the mechanism of enhanced viral protection is Ab based. Although significantly more IgG1 was induced with the targeted vaccine (Fig. 3*c*), it is likely that the overall titer of Ab, which correlated with viral neutralization (data not shown), is more relevant to protection than a particular subclass. In a repeat experiment, mice were immunized on days 0 and 14 with 200 μg of DNA and challenged with live virus 6 weeks after initial immunization. In this experiment, a similar enhancement of viral clearance with CTLA4-hIg-HA DNA to that shown in Fig. 4 was observed (data not shown). Incidentally, as with the Ab responses, the level of clearance observed with the

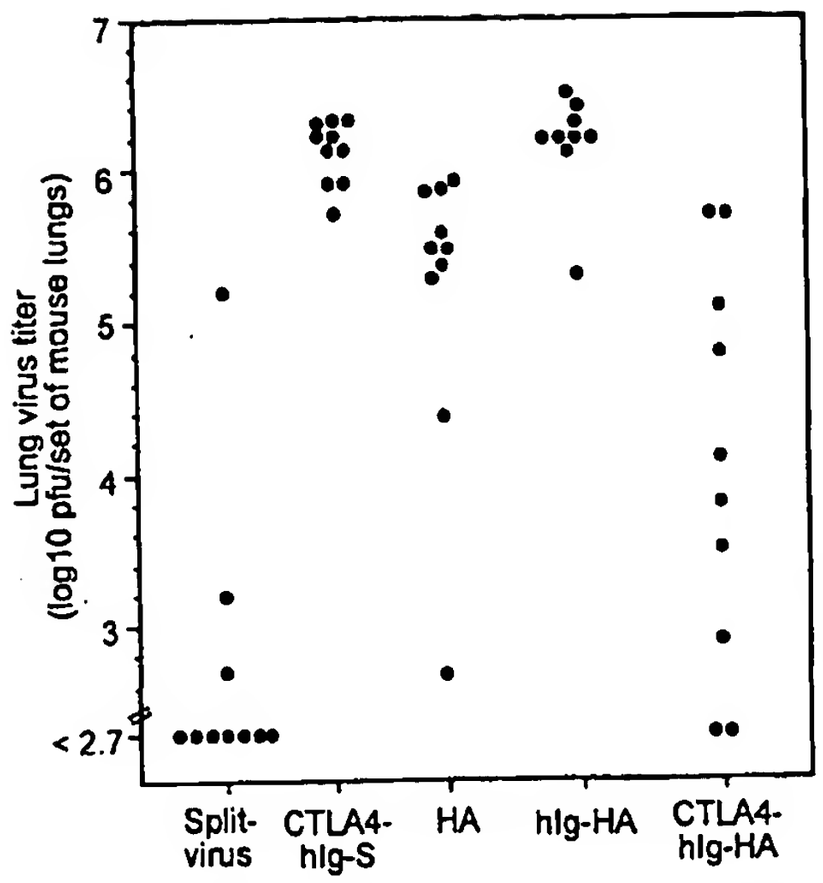

Fig. 4. Viral clearance responses induced by targeted DNA immunization were greater than those induced by nontargeted DNA immunization. Mice were immunized with 50 μg of plasmid DNA per leg in both quadriceps or with split virus as a control. After 65 days, the mice were challenged with a nonlethal dose of virus; 5 days later, the amount of infectious virus present in the lungs was determined by plaque assay. Titers for individual mice are shown.

targeted construct (CTLA4-hIg-HA) was not significantly different from that observed in mice given the split virus vaccine ($P = 0.23$), which was used as a positive vaccine control (Fig. 4). We feel, however, that this comparison is not germane, because the split virus contains HA and neuraminidase components; in addition, our strategy aims to improve on currently investigated DNA vaccine strategies through targeting so that they could be used, for example, where no current vaccine exists, rather than as a replacement for effective protein-based (or for that matter live virus) vaccines.

DNA vaccines are considered to have potential advantages because of ease of construction, ability to induce long-lasting immune responses, high temperature stability of DNA, and low production cost. Ease and speed of production could be important, particularly for those vaccine Ags that differ from one epidemic to the next; as such, the vaccines need continual change. The potency of DNA vaccines has been improved in other systems with the inclusion of immunostimulatory sequences (17), DNA-encoding cytokines (18), or costimulator molecules (19) (reviewed in ref. 14). We have shown previously that the immune response to a model Ag fused to CTLA4 could be markedly enhanced (4). However, for the progression of DNA vaccines based on the targeting strategy described herein, it was important that the crucial issues of potency of immune responses and protective efficacy be addressed (20). Our results show that targeting influenza HA to APCs can be used to greatly increase the Ab response and, more importantly, the level of protection achieved. Thus, the targeting strategy described here may be useful as a generic strategy for improving the potency and efficacy of DNA vaccines.

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IL-2 linked to a peptide from influenza hemagglutinin enhances T cell activation by affecting the antigen-presentation function of bone marrow-derived dendritic cells

Lee Faulkner, Glenn Buchan, Euan Lockhart, Lynn Slobbe, Michelle Wilson and **Margaret Baird**

Department of Microbiology, University of Otago, PO Box 56, Dunedin, New Zealand.

Keywords: antigen presentation, cytokines, dendritic cells, hemagglutinin, IL-2 receptor

Abstract

Chimeric proteins containing antigen linked to cytokines have shown some promise as vaccine candidates but little is known of their mechanism of action, particularly at the level of the antigen-presenting cell. We have investigated this using a chimeric protein in which an immunodominant T cell epitope from influenza hemagglutinin peptide (HA), recognized in the context of I-E^d, was fused to IL-2. Immature murine dendritic cells (DC) derived from bone marrow (BMDC) were used to present the chimeric protein to a T cell hybridoma with TCR specific for the HA peptide (A5 cell line). HA–IL-2 was found to induce significantly higher T cell activation than HA alone. Although the inclusion of IL-2 and HA separately did increase the response of A5 cells compared to HA alone, they were not as effective as the HA–IL-2 chimeric protein. When an antibody known to block IL-2 receptor α chain (CD25) was included, A5 activation was reduced, suggesting a role for the receptor in this process. Expression of CD25 on A5 cells was low during activation, implying that the effect was mediated by CD25$^+$ BMDC. Antigen uptake and processing of HA–IL-2 by BMDC was required since fixing BMDC, prior to antigen exposure, greatly reduced their ability to activate A5 cells. The function of CD25 on DC is currently unknown. Our results suggest this receptor may play a role in antigen uptake and subsequent T cell activation by receptor-mediated endocytosis of antigen attached to IL-2. This finding that may have implications for the development of a new generation of vaccines.

Introduction

Despite the success of many vaccines, complete protection against some pathogens has proved elusive. As a result, the potential for specific cytokines to act as vaccine adjuvants, selecting for an immune response that is appropriate to remove the pathogen or neutralize its products has been under some scrutiny. Co-delivered cytokines have had variable success owing to the short half-life and toxicity associated with the high dosages required (1). Work carried out some years ago suggested that the immunogenicity of proteins could be improved if they were fused to cytokines (2). Linking an antigenic moiety and a cytokine may prolong the half-life of the cytokine, and thereby amplify its effect on the ensuing response. Chimeric vaccines such as these are delivered to the same antigen-presenting cell (APC) which appears to be important in optimizing reactivity (3). Recent work has suggested that they may have the potential to protect against pathogens, tumours and even some hyper-immune conditions. Pneumococcal surface protein A fused to IL-2 generates antibody which, when passively transferred, confers protection against challenge with *S. pneumoniae* (4). Idiotypic proteins fused to granulocyte macrophage colony stimulating factor (GM-CSF) have been shown to immunize mice against B cell lymphomas (5). Immunization with ovalbumin fused to IL-12 has been shown to re-direct established IL-4 responses toward IFN-γ which may be applicable in the treatment of allergies (6). These vaccines also show some promise when delivered as DNA constructs (7,8).

Correspondence to: L. Faulkner

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Surprisingly little is known about the way in which fusion vaccines might operate at the cellular level. Since the fusion protein must be processed and presented it seems lik ly that, at least in part, they influence the response via their effects on APC. Dendritic cells (DC) are essential for the initiation of T cell-dependent immune responses and it is therefore appropriate that new strategies for improving the efficacy of vaccines be explored utilizing these cells. They may be crucial in determining the magnitude and the direction of the response at its earliest phase. DC express a number of cytokine receptors including those for IFN-γ, IL-1β, IL-4, IL-10, GM-CSF and tumor necrosis factor (TNF)-α (reviewed in 9). These transduce signals when they bind their respective ligands which can induce cell maturation, activation and migration. Chimeric vaccines incorporating these cytokines therefore have the potential to affect the response generated via this pathway. Interestingly, DC from various cultured tissues express the α chain (CD25) and the common cytokine γ chain but not the β chain of the IL-2 receptor (IL-2R) complex. The α chain has been detected in murine Langerhans cells (10), thymic, splenic and lung DC (11–17). The γ chain has been detected in murine splenic DC, Langerhans cells and bone marrow cells grown in GM-CSF (18). The α chain appears to contribute only to the binding affinity of the functional receptor, while the β and γ chains are involved in signal transduction (19). CD25 is markedly up-regulated on murine myeloid and lymphoid DC following exposure to GM-CSF, but as yet no defined biological function has been ascribed to this molecule since in IL-2Rα null mice, neither the maturation of DC subsets nor their capacity to present alloantigen is affected (20).

Current research in our laboratory is focused on the optimization of protective immune responses using chimeric vaccines. Here we describe experiments in which we have investigated DC-mediated processing and presentation of a chimeric protein comprising a well-defined, class II-restricted T cell epitope from influenza hemagglutinin (HA) fused to IL-2 (T–HA–IL-2). On the basis of our results we suggest that the marked increase in DC-mediated T cell activation recorded in response to T–HA–IL-2 may be attributable to enhanced antigen uptake via CD25-mediated endocytosis.

Methods

Animals

Specific pathogen-free BALB/c mice (6–10 weeks old) were obtained from the Department of Animal Laboratory Sciences, University of Otago, New Zealand.

Media, cytokines and antibodies

Bone marrow cells were cultured in DMEM supplemented with L-glutamine, streptomycin, penicillin, 50 μM 2-mercaptoethanol and 5% FCS (cDMEM). The A5 T cell hybridoma cell line was cultured in IMDM supplemented with streptomycin, penicillin, 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol, 0.5 mg/ml hygromycin B and 5% FCS. All medium and supplements were purchased from Life Technologies (Auckland, New Zealand). Recombinant murine GM-CSF was purchased from R & D Systems (Minneapolis, MN). Th phycoerythrin (PE)-conjugated AMT-13 mAb (rat anti-mouse IL-2Rα chain, CD25; IgG2a) was purchased from Sigma (Castle Hill, NSW, Australia). The isotype control antibody PE-conjugated rat IgG2a isotype antibody, 3C7 mAb (rat anti-mouse IL-2Rα chain, CD25; IgG2b) and rat IgG2b isotype control antibody were all purchased from PharMingen (San Diego, CA).

Antigens

The HA peptide (110–119) SFERFEIFPK from influenza virus A/PR/8/34 (synthesized by Macromolecular Resources, Colorado State University) was used as a test antigen. Constructs were made encoding this peptide linked to IL-2, IL-4 or IFN-γ and thioredoxin (T). Mouse RNA was isolated from splenocytes stimulated with concanavalin A and converted into cDNA using Superscript II according to the manufacturer's instructions (Life Technologies). IL-2, IL-4 and IFN-γ were amplified from mouse cDNA using PCR. The 5′ primer included sequences specific for not only murine cytokine but for HA peptide, shown in bold. The murine IL-2-specific primers were (upstream) 5′-C AAG TGA TCA **TCT TTT GAA CGT TTC GAA ATC TTC CCG AAA** GGA TCC GCA CCC ACT TCA AGC TCC-3′, (downstream) 5′-ATA TGT CGA CGA ATT CTT ATT GAG GGC TTG TTG AGA T-3′. The murine-specific IL-4 primers were (upstream) 5′-A TTT GGA TCC **TCT TTT GAA CGT TTC GAA ATC TTC CCG AAA** CAT ATC CAC GGA TGC GAC AAA AAT-3′, (downstream) 5′-T ATA AAG CTT CTA CGA GTA ATC CAT TTG CAT GAT-3′. The murine-specific IFN-γ primers were (upstream) 5′-A TTA GGA TCC **TCT TTT GAA CGT TTC GAA ATC TTC CCG AAA** GAG TAC TGC CAC GGC ACA GTC ATT GAA-3′, (downstream) 5′-A TAT AAG CTT TCA GCA GCG ACT CCT TTT CCG CTT-3′. For constructs containing just the cytokine the upstream primer did not contain the HA sequence. A construct containing just the HA peptide (110–119) and one with additional flanking sequences (94–131) were made by PCR from a DNA sequence of HA from influenza virus A/PR/8/34 kindly provided by Alistair Ramsay (University of Newcastle, Newcastle, NSW, Australia). The HA(110–119)-specific primers were (upstream) 5′-GAA TCC TCT TTT GAA CGT TTC GAA GAA ATC TTC CCG AAA TAA GCT T-3′, (downstream) 5′-CCT AGG AGA AAA CTT GCA AAG CTT TAG AAG GGC TTT ATT CGA A-3′. The HA(94–131)-specific primers were (upstream) 5′- GGA TCC GAT TTC ATC GAC TAT GAG GAG-3′, (downstream) 5′-A AGC TTA TCC TTT GGT TGT GTT GTG GTT-3′. The resulting PCR products were digested with *Bcl*I and *Sal*I for the IL-2 constructs and *Bam*HI and *Hin*dIII for all the remaining constructs, and then cloned into the thioredoxin fusion vector pET 32a (Novagen, Madison, WI). The constructs were expressed in *Escherichia coli* BL21(DE3)pLysS and the recombinant protein purified over a Ni-NTA-Agarose (Qiagen, Hilden, Germany) column. The protein concentration was measured by using the BioRad protein assay (BioRad, Auckland, New Zealand). Therefore the recombinant protein had thioredoxin at the N-terminus of HA peptide and IL-2, IL-4 or IFN-γ at the C-terminus ($T–HA_{110-119}$, $T–HA_{94-131}$, T–HA–IL-2, T–HA–IL-4, T–HA–IFN-γ).

Generation of APC

A modified method of Inaba *et al.* (21) was used for the isolation of bone marrow precursors. Briefly, the bone

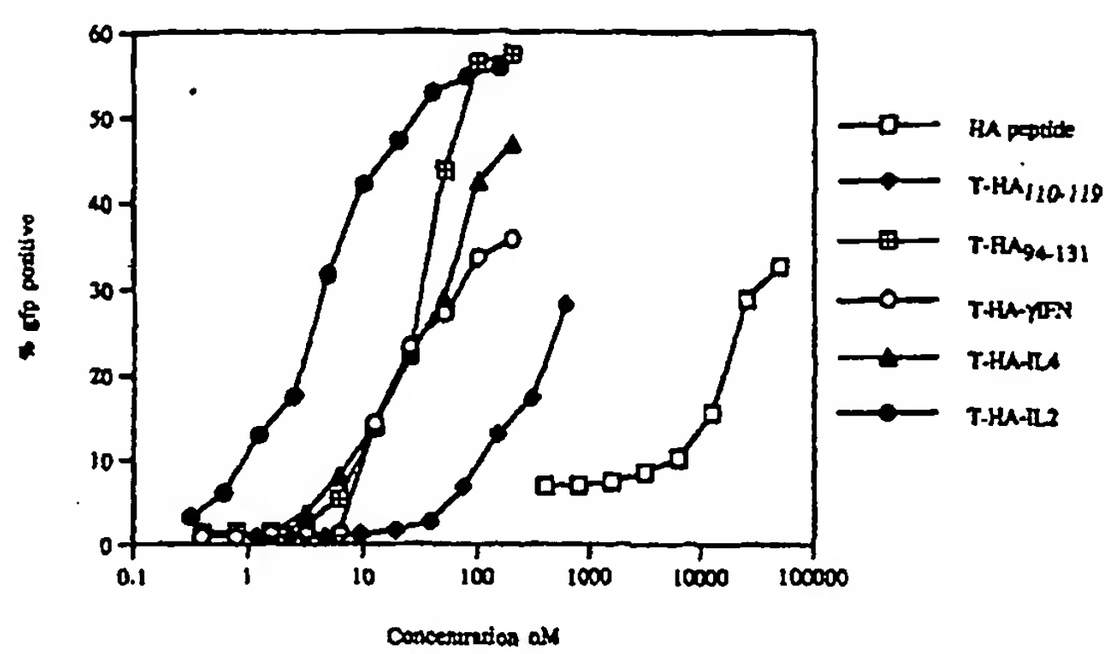


Fig. 1. Activation of A5 cells by BMDC and different forms of HA antigen. A5 cells (2×10^5) and BMDC (1×10^5) were incubated overnight with varying concentrations of antigen. Induction of GFP was analyzed by flow cytometry. The results are given as the percent GFP^+ cells. Three experiments were performed and the results of one representative experiment are shown here.

marrow was flushed from femurs and tibias with 5% FCS in PBS, the red blood cells were lysed with ammonium chloride, and the remaining cells washed 3 times. Cells were plated at 2×10^6 cells/ml in six-well plates and cultured in medium containing 20 ng/ml GM-CSF. The cultures were fed on day 4 and on day 6 by replacing ~75% of the medium. On days 6–7 non-adherent cells were harvested as bone marrow-derived DC (BMDC). BMDC cultured in GM-CSF show an immature phenotype and are capable of taking up both soluble and particulate antigen (22). Approximately 50–60% of the cells are MHC class II^+, 40–50% $CD80^+$ and 25–35% $CD86^+$. Resident macrophages were harvested from BALB/c mice by peritoneal lavage using 5% FCS, 5 U/ml heparin in PBS and then used on the same day.

Assay for T cell activation

T c ll activation was measured using the A5 cell line which was derived from the 14-3-d T cell hybridoma expressing TCR specific for the influenza HA peptide 110–120 presented in the context of MHC class II I-E^d molecules (23). These cells have been transfected with a construct containing a triple NF-AT promotor linked to green fluorescent protein (GFP). Upon activation these cells produce GFP which can be detected by flow cytometry. A5 cells were subcultured 1:10 the day before use. To induce activation 2×10^5 A5 cells and 1×10^5 BMDC or peritoneal macrophages were added to 12-mm diameter, round-bottomed polystyrene tubes and pulsed overnight with varying concentrations of HA peptide, T–HA chimeric proteins or thioredoxin alone. Assays were performed using cDMEM. The cells were washed once and analyzed by flow cytometry using a FACSCalibur (Becton Dickinson, Mountain View, CA). A minimum of 10,000 events per sample were analyzed using CellQuest software. Fluorescence data was collected with logarithmic amplification.

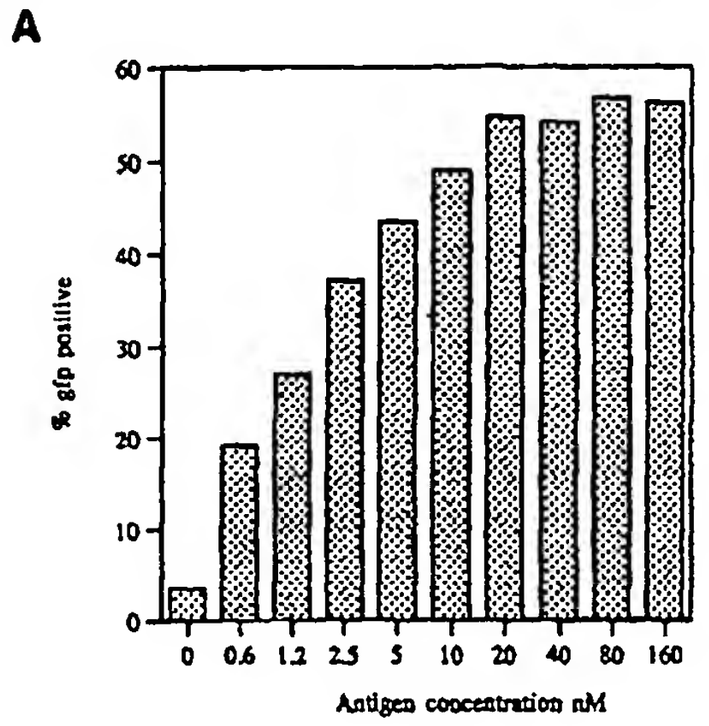
Analysis of the role of BMDC in T cell activation using fusion proteins

BMDC were exposed to antigen for 2 h at 37°C, wash d twice and mixed with A5 cells overnight. Before and after antigen exposure BMDC were treated with 1% paraformaldehyde for 1 min at room temperature and washed twice with PBS. BMDC alone and BMDC together with A5 cells were exposed to antigen overnight and then stained with PE-conjugated AMT-13 for 40 min on ice (1/200 dilution). Untreated peritoneal macrophages and A5 cells were stained in the same way. The degree of expression of CD25 (IL-2Rα) was then measured by flow cytometry. To test the effect of blocking CD25 on T cell activation a mAb known to block receptor function was used (24). BMDC and A5 cells were incubated separately for 40 min on ice with 3C7 mAb or isotype-matched control at 5 μg/ml. The BMDC were then exposed to antigen for 2 h at 37°C. The BMDC and A5 cells were then mixed together, washed 3 times, and incubated overnight. The cells were washed once and analyzed by flow cytometry.

Results

Linking IL-2 to the HA antigen greatly increased the activation of A5 cells compared to HA antigen alone

A5 cells and BMDC were mixed in a 2:1 ratio and incubated with antigen overnight. Activation of A5 cells by antigen resulted in induction of GFP which was detected by flow cytometry. All forms of the HA antigen caused activation of the A5 cells, i.e. HA peptide alone and the chimeric proteins: $T–HA_{110–119}$, $T–HA_{94–131}$, T–HA–IL-2, T–HA–IL-4 and T–HA-IFN-γ (Fig. 1). The percent of GFP^+ A5 cells and their mean fluorescence increased with increasing antigen concentration (Figs 1 and 2). The theoretical maximum level of A5 activation was 66%, although in practise the actual maximum was slightly lower and usually between 50–60%. When the percent GFP^+ cells was maximal then mean fluorescence was a more sensitive indicator of A5 activation as it maintained its dose responsiveness (Fig. 2). The presence of the HA antigen was absolutely necessary for A5 cell activation since exposure to 200 nM T–IL-2, T–IL-4, T–IFN-γ, thioredoxin or medium alone resulted in <5% of A5 cells becoming activated.



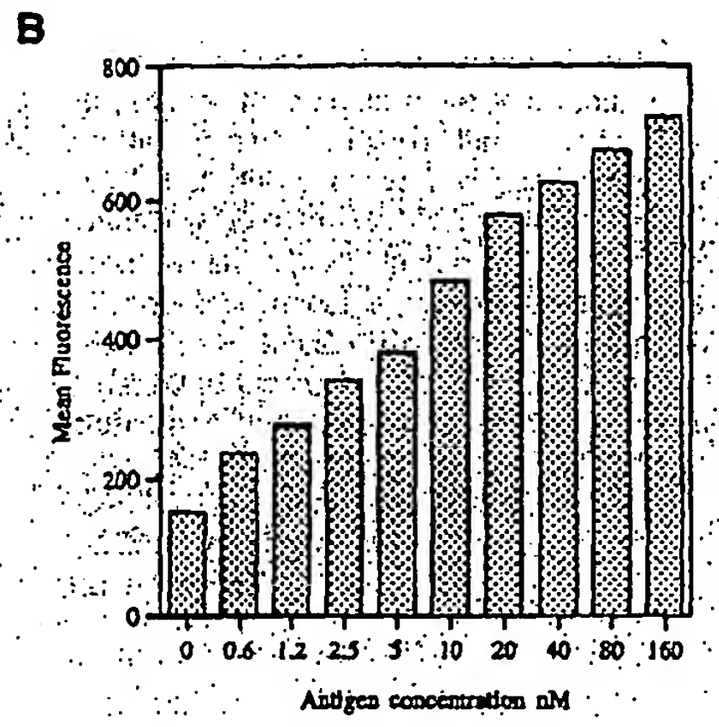


Fig. 2. Activation of A5 cells by T–HA–IL-2. A5 cells (2×10^5) and BMDC (1×10^5) were incubated overnight with varying concentrations of T–HA–IL-2. Induction of GFP was analyzed by flow cytometry and the results are given as the percent GFP^+ cells and the mean fluorescence of the GFP^+ cells. Three experiments were performed and the results of one representative experiment are shown here.

This result also shows that the A5 cells were not activated by bacterial products, such as lipopolysaccharide, that may be present in the fusion protein preparations. Since all fusion proteins were prepared in the same way, any contaminating bacterial products would be present in all the different preparations.

The concentration of antigen required to activate A5 cells depended on the form of the antigen available to the APC. When BMDC and A5 cells were exposed to HA peptide then cell activation was detected over a concentration range of 3–50 μM (Fig. 1). When the peptide was produced as a fusion protein with thioredoxin, $T–HA_{110–119}$, then less antigen was required for cell activation, i.e. 75–600 nM (Fig. 1). This trend continued when additional flanking sequences wer added to the HA peptide, i.e. $T–HA_{94–131}$, and when IL-2, IL-4 and IFN-γ wer linked to the HA(110–119) antigen. In these forms an antigen concentration of 1–150 nM was able to activate A5 cells (Fig. 1). However, at concentrations <10 nM the T–HA–IL-2 fusion protein was by far the most effective at activating A5 cells.

Thus, the T–HA–IL-2 form of the HA antigen was capable of activating A5 cells at 1000-fold less concentration than the HA peptide when presented by BMDC. Linking the antigen to another cytokine, e.g. IL-4, was better than the T–HA or peptide alone but not as effective as linking the antigen to IL-2.

The type of APC used was also important with respect to the presentation of the chimeric proteins. When resident peritoneal macrophages were used instead of BMDC then a slightly different pattern of A5 activation was seen. T–HA–IL-2 and T–HA–IL-4 gave similar levels of activation, and were more effective at lower concentrations than $T–HA_{94–131}$ and T–HA-IFN-γ (Fig. 3). All these chimeric proteins were more effective than $T–HA_{110–119}$ as seen with BMDC.

Adding the IL-2 and HA antigen separately was not as effective at activating A5 cells as linking the two in a chimeric protein

To investigate whether the physical linkage of the cytokine and antigen was necessary for this increased sensitivity we compared A5 cell activation by T–HA–IL-2 to activation by T–HA and IL-2 added separately. BMDC and A5 cells were exposed to three different concentrations of T–HA in the presence of three different concentrations T–IL-2. Adding IL-2 separately did increase cell activation by T–HA but was not as effective as linking the two in the fusion protein, T–HA–IL-2 (Fig. 4). T–IL-2 alone did not activate the A5 cells (< 1% GFP^+), whereas between 19 and 54% of A5 cells were GFP^+ in the presence of BMDC incubated with T–HA–IL-2 and T–HA alone and in combination with T–IL-2.

Paraformaldehyde inhibited the ability of BMDC to activate A5 cells

To investigate whether antigen processing was necessary for A5 cell activation, BMDC were treated with paraformaldehyde before and after antigen exposure. The antigen concentration used was selected to give similar levels of A5 activation by the three different antigens. Treatment of BMDC with paraformaldehyde decreased A5 cell activation by HA peptide, T–HA and T–HA–IL-2 (Fig. 5). This shows that antigen processing was required for the presentation of the HA epitope from both T–HA and T–HA–IL-2 and for the peptide itself.

Inclusion of an antibody blocking CD25 inhibited T cell activation via BMDC

Even though IL-2 alone did not activate A5 cells, it was possible that the IL-2 component of the antigen was having a direct effect on the APC and A5 cells. To investigate this possibility we measured IL-2Rα (CD25) expression on both BMDC and A5 cells before and after antigen exposure. The antigen concentration used was selected to give similar levels of A5 activation by the two different antigens. Expression of CD25 could not be detected on unstimulated A5 cells (Table 1). After antigen activation th level of CD25 expression on GFP^+ A5 cells was very low, sugg sting that the

T–HA–IL-2 construct had little or no direct affect on receptor expression of these cells (Table 1). Furthermore, activation by both T–HA and T–HA–IL-2 resulted in similar levels of CD25 expression, suggesting that expression was not mediated directly by IL-2.

BMDC expressed low levels of CD25 and expression was increased with exposure to T–HA and T–HA–IL-2 (Table 1), $T–HA_{94–131}$, T–HA–IL-4 and T–HA-IFN-γ (data not shown). This suggested that the IL-2 component of the antigen was not directly responsible for CD25 up-regulation but was probably indicative of DC maturation which occurs after antigen uptake (11,13,15). However, BMDC express much higher levels of CD25 compared to A5 cells which suggests that this receptor may have a role in antigen uptake and presentation of T–HA–IL-2 by BMDC. Expression of CD25 could not be detected on resident peritoneal macrophages (0.2% positive, 5.5

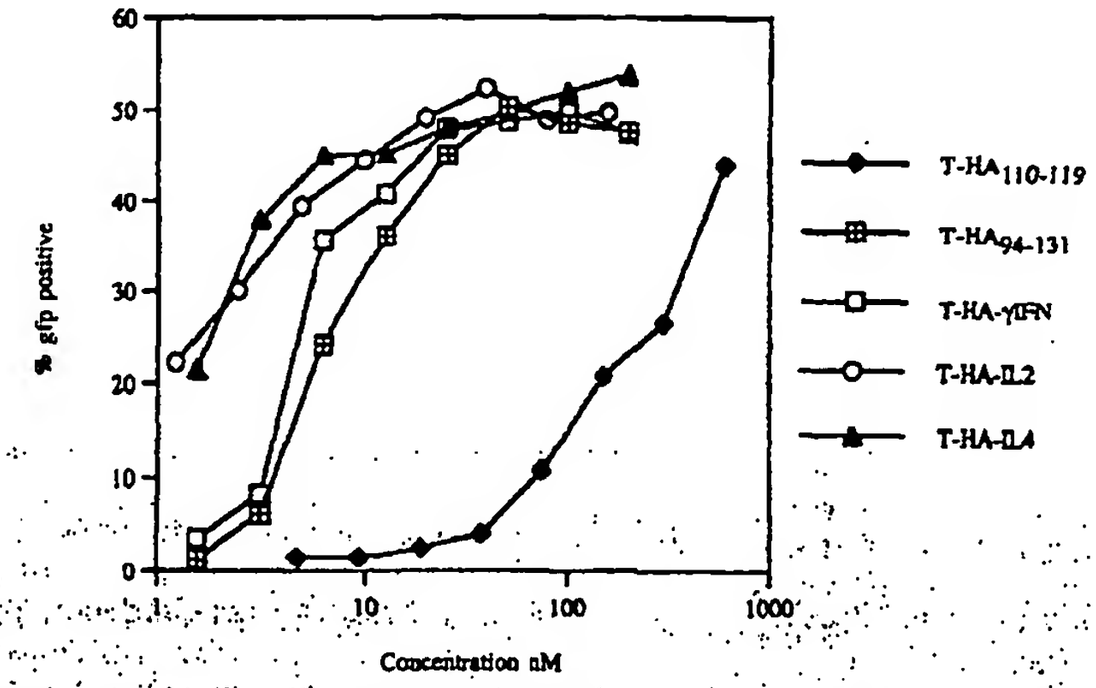


Fig. 3. Activation of A5 cells by peritoneal macrophages and different forms of HA antigen. A5 cells ($2×10^5$) and resident peritoneal macrophages ($1×10^5$) were incubated overnight with varying concentrations of antigen. Induction of GFP was analyzed by flow cytometry. The results are given as the percent GFP^+ cells. Three experiments were performed and the results of one representative experiment are shown here.

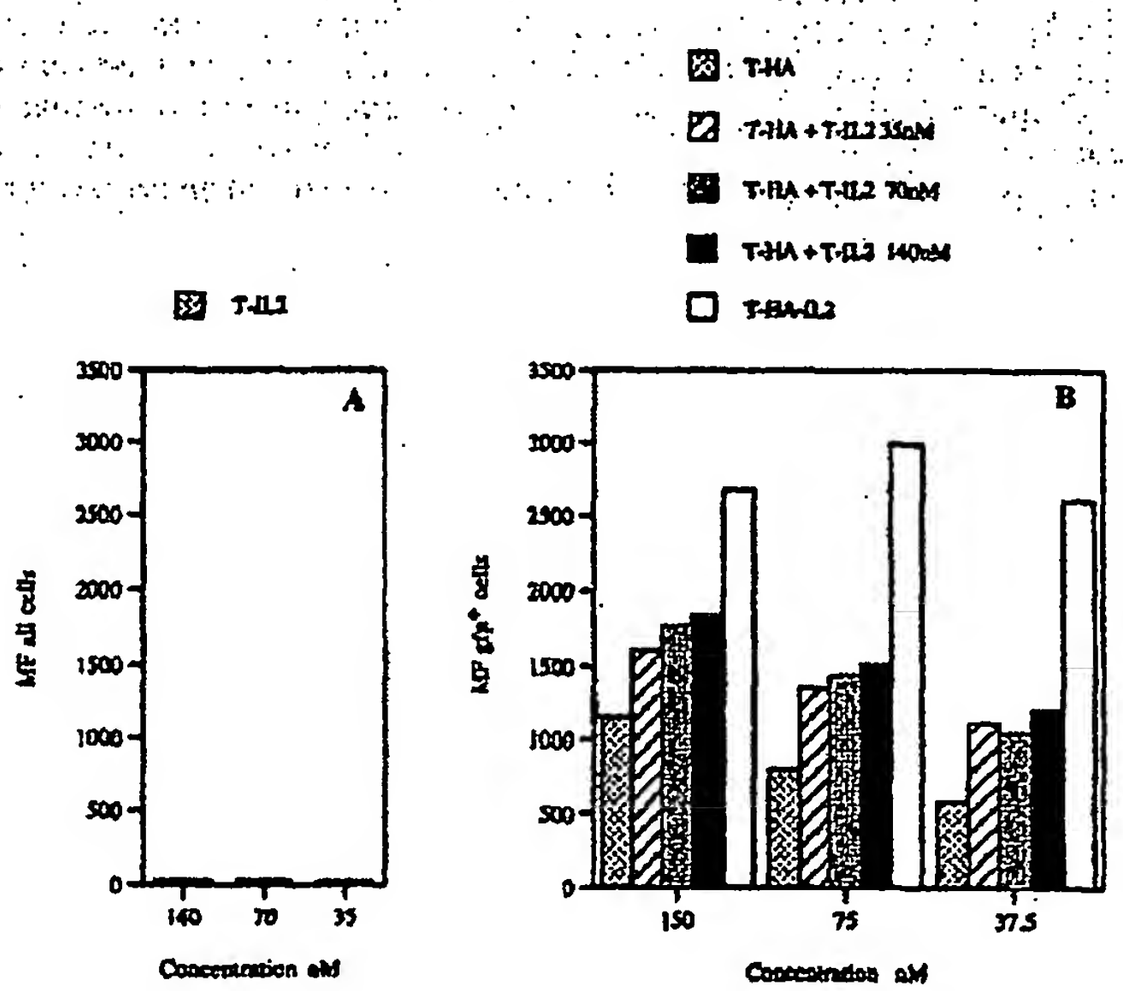


Fig. 4. Linking IL-2 to HA peptide is more effective at activating A5 cells than adding them separately. A5 cells ($2×10^5$) and BMDC ($1×10^5$) were incubated overnight with (A) T–IL-2 alone, (B) T–HA–IL-2 and T–IL-2 alone or with T–HA and T–IL-2 together. Induction of GFP was analyzed by flow cytometry. The results are given as the mean fluorescence of (A) all the cells or (B) GFP^+ cells. Three experiments were performed and the results of one representative experiment are shown here.

mean fluorescence; isotype control 0.3% positiv , 5.6 mean fluorescence).

The role of CD25 was further investigated by treating BMDC and A5 cells separately with an antibody known to block the receptor. BMDC were exposed to antigen for 2 h, in the presence of antibody, before being added to the A5 cells and incubated overnight. Activation of A5 cells by T–HA–IL-2 was decreased when BMDC were treated with CD25 antibodies but not when A5 cells were so treated. No such decrease was observed in the presence of the control antibody or when T–HA_{94-131} was used as the antigen (Fig. 6).

Discussion

We have shown that the form of the antigen supplied to the APC is important in determining the T cell response. Peptide was the least effective form of the HA antigen, whereas fusing the peptide to thioredoxin and cytokines was more effective than peptide alone in activating T cells. The presence of thioredoxin and cytokines may have enhanced the effectiveness of the HA antigen by providing epitope protection, by enhancing antigen uptake or by enhancing the subsequent processing of the HA antigen.

By flanking both ends of the HA epitope the thioredoxin and cytokine may protect the epitope from destructive degradation allowing it to be more efficiently processed into free peptide and presented to the T cell. This explanation is also consistent with the intermediate stimulatory capacity of the T–HA fusion protein where only one of the two ends of the peptide

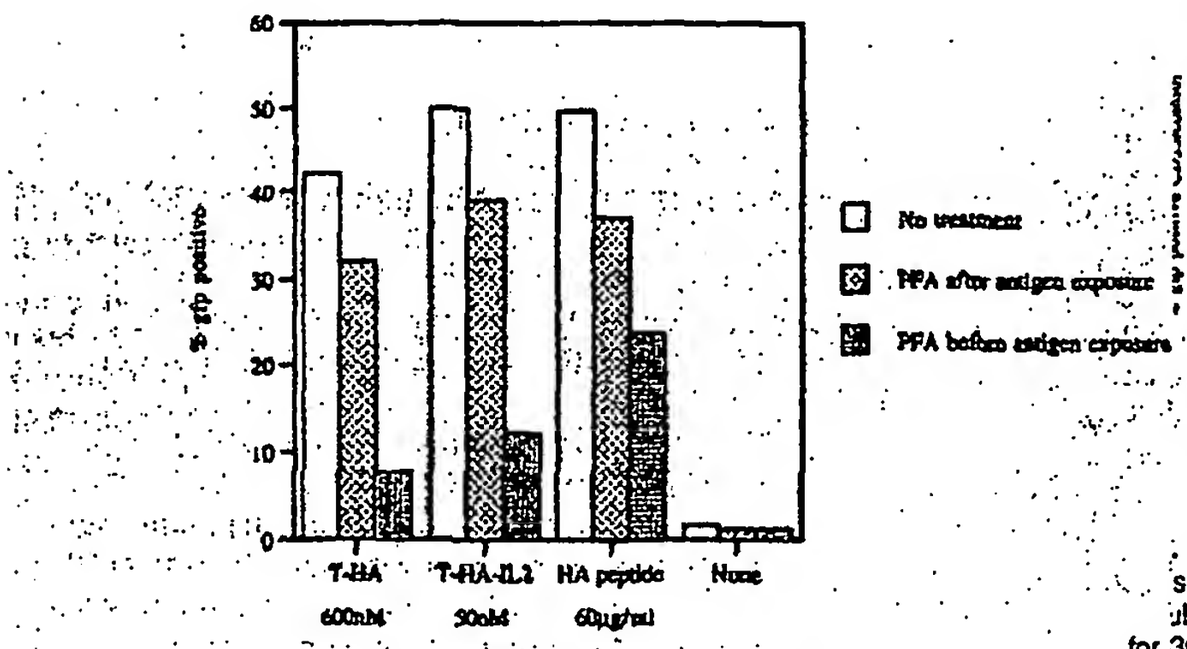


Fig. 5. Treatment of BMDC with paraformaldehyde prevents presentation of HA to A5 cells. BMDC (1×10^5) were fixed with 1% paraformaldehyde before and after antigen exposure for 2 h at 37°C. A5 cells (2×10^5) were added and incubated overnight. Induction of GFP was analyzed by flow cytometry. The results are given as the percent GFP^+ cells. Three experiments were performed and the results of one representative experiment are shown here.

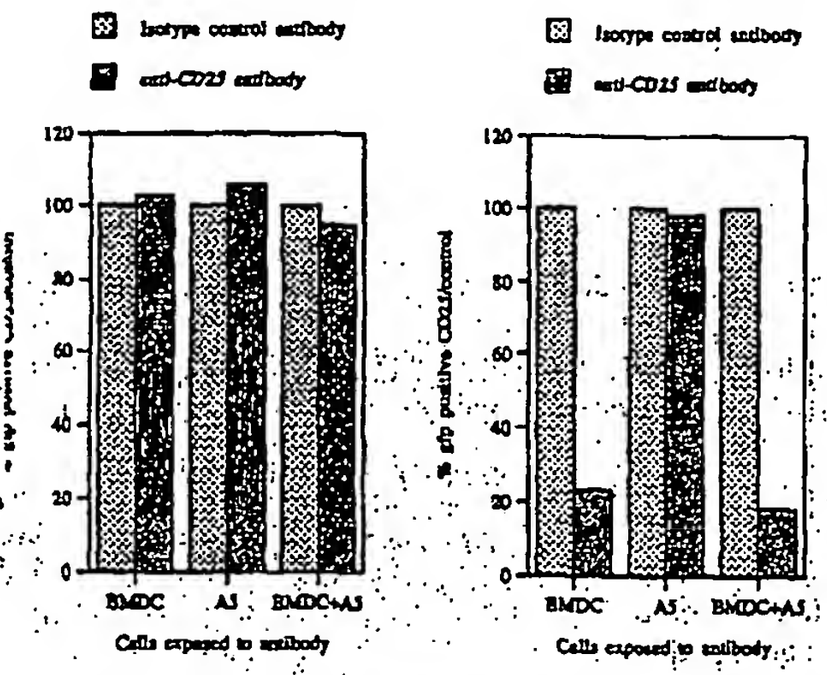


Fig. 6. Addition of anti-CD25 antibodies decreases activation of A5 s by T–HA–IL-2. A5 cells (2×10^5) and BMDC cells (1×10^5) were ubated separately with 3C7 or isotype-matched control antibody for 30 min at 4°C. BMDC were then incubated with 50 nM of antigen for 2 h at 37°C. BMDC and A5 cells were washed, added together and incubated overnight. Induction of GFP was analyzed by flow cytometry. The percent GFP^+ cells in the presence of the 3C7 mAb was calculated with respect to percent GFP^+ cells in the presence of the isotype-matched control which was assigned a value of 100%. Three experiments were performed and the results of one representative experiment are shown here.

Table 1. T–HA–IL-2 is not acting directly on A5 cells to cause activation

Antigen activation of A5 cells			CD25 expression on		
Antigen	GFP^+ A5 cells		GFP^+ A5 cells	BMDC	
	%	Mean fluorescence	%	%	Mean fluorescence
600 nM T–HA	60.1	1678.8	2.6 (0.1)	20.7 (0.5)	52.1 (10.7)
300 nM T–HA	58.3	1352.2	1.5	17.9	47.1
150 nM T–HA	50.8	918.1	1.2	15.6	41.9
10 nM T–HA–IL-2	57.0	2149.1	2.9 (0.2)	21.7 (1.6)	54.1 (15.7)
5 nM T–HA–IL-2	58.0	2206.9	2.3	10.0	33.3
2.5 nM T–HA–IL-2	52.2	1928.4	1.7	12.0	36.6
None	1.0	[11.5][a]	0.0 (0.0)	2.9 (0.8)	37.7 (17.0)

[a]Mean fluorescence of all A5 cells.

is protected. However, this explanation cannot account for the enhanced T cell activation by T–HA–IL-2 when presented by BMDC, and by both T–HA–IL-2 and T-HA–IL-4 when presented by resident peritoneal macrophages.

Antigen processing of the fusion proteins was clearly required because fixing BMDC dramatically impaired their ability to stimulate T cells. However, paraformaldehyde treatment affected the processing of both T–HA and T–HA–IL-2 to a similar degree. This implies that enhanced antigen uptake may be largely responsible for the difference in effectiveness of these two forms of the HA antigen. Indeed, preliminary experiments using labeled fusion proteins show that uptake of T–HA–IL-2 is higher than that of T–HA (unpublished observations).

An interesting difference emerged with the T–HA–IL-2 and T–HA–IL-4 fusion proteins showing that the APC was also important. Investigations into why T–HA–IL-2 and T–HA–IL-4 fusion proteins were the most effective form of the antigen at activating T cells when presented by resident peritoneal macrophages are currently underway.

In this paper we have concentrated on trying to explain why the T–HA–IL-2 fusion protein was the most effective form of the antigen at activating T cells when presented by BMDC. Our investigations suggest this difference was probably associated with CD25 expression on BMDC rather than on the responding T cell. A5 cells do not express CD25 and it seems unlikely that the small increase in CD25 expression seen on activated A5 cells could be responsible for the large difference in antigen sensitivity we detected. On the other hand, BMDC do express CD25 and the level of expression increased with antigen exposure.

The low-affinity form of the IL-2R, comprising α and γ chains in the absence of the β chain, has been detected on DC isolated from different sources, *i.e.* murine spleen, thymus, lymph nodes and skin (10–18). The relatively low level of expression we observed on BMDC compared to DC extracted from lymphoid tissues can be explained with reference to the heterogeneous nature of DC and the different stages of maturation of DC derived from different tissues. Indeed the proportion of $CD25^+$ cells is consistent with that reported by Lutz *et al.* (25), where DC were also generated under the influence of GM-CSF.

Immature DC can internalize soluble exogenous antigens by both fluid-phase uptake through macropinocytosis or by receptor-mediated uptake (reviewed in 26–28) via the mannose receptor, DEC 205 (29), and Fc receptors (30,31). The antigens are delivered to endocytic/lysosomal compartments for processing and loading on to MHC class II molecules. We suggest that CD25 on DC may also be involved in receptor-mediated antigen uptake. If the same antigen can be taken up by both macropinocytosis and receptor-mediated uptake then the density of antigen on the cell surface is likely to be greater. This will result in greater T cell activation. It has been demonstrated that mannose receptor-mediated antigen uptake was 100-fold more efficient at antigen presentation to T cells (32). The efficiency of receptor-mediated uptake in turn depends on whether the receptor is used once like the Fc receptors (33) or wheth r it is constitutively recycled like the mannose receptor (34). It is not known whether CD25 is constitutively recycled in DC. However, in T cells the α, β and γ chains of the IL-2R have been shown to localize to different subcellular compartments and undergo different fates. Following IL-2 binding, the α chain was found in an early endosomal compartment and subsequently recycled to the plasma membrane, whereas both the β and γ chains were found in the late endosomal compartment and subsequently degraded (35,36). If CD25 acts in a similar manner to the mannose receptor then even a low of expression could have a marked effect on antigen uptake.

The physical linkage of the peptide and cytokine was required for the enhanced activity, suggesting that delivery at the same time and to the same APC was important. The importance of co-delivery has been reported by others using DNA constructs—those encoding antigen together with cytokine were found to be more effective immunogens than DNA encoding either the antigen or the cytokine alone (7,37).

Previous work by others using IL-2Rα null mice suggested that this chain was not involved in DC differentiation since these mice had a full complement of cells. Neither could this be implicated in the presentation of alloantigen since APC from these mice were capable of stimulating mixed lymphocyte responses that were indistinguishable in magnitude from those generated using stimulator cells from wild-type mice. The authors did point out that it was possible that CD25 may play a role in antigen acquisition. We suggest that our results support a model in which antigen fused to IL-2 is taken up by DC more efficiently using receptor-mediated endocytosis, resulting in greater antigen density on the cell surface or possibly a greater degree of DC activation. This in turn is reflected in enhanced stimulatory capacity. This suggestion is supported by two findings. Firstly, that delivery of equivalent amounts of IL-2 and the peptide separately failed to increase T cell activation to the same degree and secondly, that blocking CD25 on DC with an antibody during antigen exposure resulted in a significant decrease in T cell activation.

It has been reported that the absence of the β chain precludes signal transduction through the IL-2R, resulting in a non-functional receptor on DC (19,20). However, a recent report by Fukao and Koyasu (16) has demonstrated that mature murine splenic DC are capable of responding to IL-2, arguing for a functional IL-2R. IL-2 augmented the production of IFN-γ by these cells when they were stimulated with IL-12 or by CD40 and MHC class II cross-linking. Furthermore, this enhancing effect was blocked by CD25 antibodies. The role of the β chain in this process remains uncertain since the authors failed to detect IL-2Rβ chain on the cell surface but did detect IL-2Rβ chain mRNA. This finding opens the possibility that T–HA–IL-2 could have other affects on DC function by signaling directly through the IL-2R.

The physiological relevance of antigen acquisition via the CD25 is unclear. It is not inconceivable that some viruses express receptors homologous to mammalian CD25. Various pox viruses have been shown to express a range of cytokine receptor homologues. These may block the delivery of cytokines detrimental to virus survival (38). Many of these receptors are soluble but a few, such as vaccinia virus TNFR, are found in membrane-bound form (39). This suggests a mechanism for host counter-attack sinc binding IL-2 to a surface receptor such as this could render a virus susceptible to uptake via CD25 on the APC surface. Alternatively, CD25-mediated

antigen acquisition may simply be an unexpected use of a receptor designed for other physiological purposes. For instance, there is some evidence to suggest that IL-2 may be important in the development of DC from progenitor cells in human cord blood (40), although this does not seem to be the case in mice. Irrespective of these speculations, there remains the exciting possibility that our results may be exploited in the development of new vaccination strategies by targeting fusion proteins to receptors on APC.

Acknowledgements

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Abbreviations

APC	antigen-presenting cell
BMDC	bone marrow-derived dendritic cell
DC	dendritic cell
GFP	green fluorescent protein
GM-CSF	granulocyte macrophage colony stimulating factor
HA	hemagglutinin peptide
IL-2R	IL-2 receptor
PE	phytohemagglutinin
T	thioredoxin
TNF	tumor necrosis factor

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(2)

The Ch14.18-GM-CSF Fusion Protein Is Effective at Mediating Antibody-dependent Cellular Cytotoxicity and Complement-dependent Cytotoxicity *in Vitro*[1]

Ayse Batova, Arvid Kamps, Stephen D. Gillies, Ralph A. Reisfeld, and Alice L. Yu[2]

Department of Pediatrics, Division of Pediatric Hematology/Oncology, University of California, San Diego, San Diego, California 92103-8447 [A. B., A. L. Y.]; Isala Clinics, Weezenlanden Hospital, Department of Pediatrics, 8000 GM Zwolle, the Netherlands [A. K.]; Fuji ImmunoPharmaceuticals Corporation, Lexington, Massachusetts 02173 [S. D. G.], and Department of Immunology, Scripps Research Institute, La Jolla, California 92037 [R. A. R.]

ABSTRACT

Granulocyte/macrophage-colony stimulating factor (GM-CSF) is very effective at enhancing antibody-dependent cellular cytotoxicity (ADCC) mediated by granulocytes and monocytes. Recently, a fusion protein consisting of GM-CSF and chimeric human/mouse anti-ganglioside G_{D2} antibody Ch14.18 (Ch14.18-GM-CSF) has been generated to improve the effectiveness of immunotherapy by directing GM-CSF to the tumor microenvironment and prolonging its relatively short half-life. In this study, we examined the ability of this fusion protein to enhance the *in vitro* killing of G_{D2}-expressing human neuroblastoma cells by granulocytes and mononuclear cells, as well as by complement. The Ch14.18-GM-CSF fusion protein was equally effective as the combination of equivalent amounts of free Ch14.18 and GM-CSF in mediating the killing of NMB7 neuroblastoma cells by granulocytes from seven of eight neuroblastoma patients. The fusion protein was also equally effective as the combination of Ch14.18 and GM-CSF in mediating ADCC by neuroblastoma patients' mononuclear cells. In addition, the fusion protein was as effective as Ch14.18 alone in directing complement-dependent cytotoxicity against NMB7 cells. Our results demonstrate that the biological activities expressed by ADCC and complement-dependent cytotoxicity of both monoclonal antibody Ch14.18 and GM-CSF are retained by the Ch14.18-GM-CSF fusion protein and lend further support for future clinical trials of this fusion protein in patients with neuroblastoma.

INTRODUCTION

Advances in cancer therapy in the last three decades have transformed the majority of childhood cancers from uniformly fatal diseases to largely curable illnesses. Unfortunately, the outcome of advanced stage neuroblastoma, which comprises more than one-half of all neuroblastoma, remains dismal despite surgery, radiation, intensive chemotherapy, and bone marrow transplantation. Clearly, new therapeutic strategies for the treatment of neuroblastoma are urgently needed. A promising approach is targeted therapy with mAbs[3] directed against human tumor-associated antigens. In this regard, immunotherapy with mAbs directed against the neuroblastoma-associated antigen, disialoganglioside G_{D2}, has been actively pursued (1–3). The G_{D2} antigen is ideal for mAb-mediated therapy of neuroblastoma because it is expressed at high density in the vast majority of human neuroblastoma cells but is absent in normal tissues excluding neurons, skin melanocytes, and peripheral pain fibers, where it is poorly expressed (4, 5). To minimize immunogenicity, a human-mouse chimeric antibody directed against ganglioside G_{D2} (Ch14.18) was developed by fusing the cDNA sequences encoding the constant portion of human γ1 heavy chain and κ light chain with those encoding the variable portions of immunoglobulin from the murine hybridoma 14.18 (6). Ch14.18 was demonstrated to be very effective in mediating ADCC against neuroblastoma cells in the presence of human granulocytes and mononuclear cells (7), as well as in directing complement against neuroblastoma and melanoma (8). Phase I clinical trials of Ch14.18, conducted by us and others in children with neuroblastoma, revealed that Ch14.18 had therapeutic efficacy and was fairly well tolerated with proper supportive care (9, 10).

Many cytokines enhance effector cell functions, particularly in mediating ADCC (11–14). Our recent studies demonstrated that GM-CSF enhances anti-G_{D2}-mediated ADCC by granulocytes of normal individuals as well as those of neuroblastoma patients (15). These and other encouraging results have prompted the use of GM-CSF, in conjunction with mAbs in clinical trials, for the treatment of colorectal carcinoma (16, 17) and neuroblastoma (18). GM-CSF augmented ADCC activity of mononuclear cells and granulocytes against colorectal

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[2] To whom requests for reprints should be addressed, at Division of Pediatric Hematology/Oncology, UCSD Medical Center, 200 West Arbor Drive, San Diego, CA 92103-8447.

[3] The abbreviations used are: mAb, monoclonal antibody; ADCC, antibody-dependent cellular cytotoxicity; CDC, complement-dependent cytotoxicity; GM-CSF, granulocyte/macrophage-colony stimulating factor; IL-2, interleukin 2; TNF, tumor necrosis factor; LU, lytic unit(s); HAMA, human antimouse antibody.

Table 1 Antibody-dependent lytic activity of GM-CSF-stimulated granulocytes and mononuclear cells

Granulocytes and mononuclear cells isolated from peripheral blood or bone marrow of neuroblastoma patients were used as effector cells against NMB7 human neuroblastoma cells in the presence of Ch14.18, Ch14.18 plus GM-CSF, or Ch14.18-GM-CSF fusion protein. Whether added individually or as a fusion protein, the Ch14.18 concentration was maintained at 1 μg/ml, and the GM-CSF concentration was 260 ng/ml. In the absence of Ch14.18 and GM-CSF, lytic activity of granulocytes of all eight neuroblastoma patients was equal to background (0.2 ± 0.5 LU), and that of mononuclear cells of patient U13 averaged 6.9 ± 1.8 LU, in four independent experiments. Specific target cell lysis by Ch14.18 or Ch14.18-GM-CSF fusion protein at 1 μg/ml, in the absence of effector cells, was 1.0 ± 1.4%.

Patient characteristics			Lytic activity (LU at 20%)/10^6 effector cells		
			Granulocyte ADCC		
No.	Site of tumor[a]	BMT	Ch14.18	Ch14.18 + GM-CSF	Ch14.18-GM-CSF fusion protein
P1006	BM, bone	No	55.8 ± 5.6	81.8 ± 10.8	114 ± 13
P1009	BM, chest	Yes	9.1 ± 1.7	14.4 ± 3.3	14.7 ± 3.4
P1010	BM, bone	Yes	10.0 ± 1.0	8.6 ± 0.7	7.9 ± 1.4
P1011	BM, chest	No	10.7 ± 3.7	31.3 ± 6.2	21.7 ± 4.8
P1013	BM, bone	Yes	7.0 ± 2.0	5.2 ± 1.3	7.2 ± 1.7
P1014	Chest	No	44.4 ± 2.5	107 ± 12.0	132 ± 6.0
P1015	BM, bone	No	0.8 ± 0.2	9.4 ± 0.9	2.8 ± 0.2
U13	BM	Yes	5.1 ± 0.6	17.0 ± 2.2	16.5 ± 1.7
U13[b]			3.2 ± 1.3	26.8 ± 4.8	22.1 ± 3.4
			Mononuclear cell ADCC		
U13			46.6 ± 4.2	67.6 ± 2.8	59.6 ± 1.8
U13			42.6 ± 4.5	59.0 ± 4.4	64.8 ± 6.4

[a] BM, bone marrow; BMT, bone marrow transplant.
[b] Granulocytes and mononuclear cells isolated from bone marrow.

carcinoma cells (16, 17) and neuroblastoma (18), respectively. In both clinical trials, the use of GM-CSF in conjunction with tumor antigen-specific mAbs demonstrated therapeutic efficacy. In view of the effectiveness by which cytokines stimulate the proliferation of various effector cells and enhance their ADCC activities, efforts have been made to target cytokines to the tumor microenvironment by using genetically engineered fusion proteins consisting of cytokines and mAbs directed against tumor-associated antigens. For example, Ch14.18 has been fused to IL-2, GM-CSF, TNF-α, and TNF-β (19–21). These fusion proteins were shown to bind G_{D2} on neuroblastoma and melanoma cells, and Ch14.18-IL2 was reported to stimulate $CD8^+$ T-cell-mediated killing of these tumors in syngeneic mouse tumor models. Previously, the Ch14.18-GM-CSF fusion protein was found to bind purified G_{D2} antigen similarly as Ch14.18 but was five times less active than recombinant GM-CSF in stimulating the proliferation of GM-CSF-dependent cells (19). However, a more important question to answer is whether fusion of GM-CSF to mAb Ch14.18 interferes with the well-known biological activities of Ch14.18 and GM-CSF such as ADCC and CDC.

Here, we investigated for the first time the ability of the Ch14.18-GM-CSF fusion protein to stimulate ADCC by granulocytes and mononuclear cells of neuroblastoma patients and to mediate complement-dependent lysis of human neuroblastoma cells. Our results indicated the Ch14.18-GM-CSF fusion protein to be equally effective as a mixture of equivalent amounts of Ch14.18 and GM-CSF in mediating ADCC by granulocytes and mononuclear cells. This finding indicates that the ability of Ch14.18 to mediate ADCC, and of GM-CSF to enhance ADCC, is preserved in the fusion protein. At concentrations up to 0.2 μg/ml, the Ch14.18-GM-CSF fusion protein was equally effective as mAb Ch14.18 in directing complement-dependent lysis of neuroblastoma cells.

MATERIALS AND METHODS

Cell Lines and Antibodies. The neuroblastoma cell line NMB7 was graciously provided by Dr. Shuen-Kuei Liao, Chang Gung Medical College, Tao-Yuan, Taiwan. The human-mouse chimeric anti-G_{D2} antibody, Ch14.18 (6), and the Ch14.18-GM-CSF fusion protein (19) were developed and characterized as described previously.

Patient Population. The patient population consisted of eight high-risk neuroblastoma patients who had recurrent or refractory disease after at least one regimen of multiagent chemotherapy and autologous bone marrow transplant in four or eight patients (Table 1).

Isolation of Granulocytes and Mononuclear Cells. Granulocytes were isolated from eight neuroblastoma patients characterized in Table 1. Briefly, patients' heparinized whole blood or bone marrow were centrifuged on a Ficoll-Paque density gradient at 400 × g for 30 min. The resulting pellet, consisting of RBCs and granulocytes, was washed with RPMI 1640, resuspended in hemolytic buffer (0.15 M NH_4Cl and 0.01 M $NaHCO_3$), and incubated for 2 min at 37°C to lyse RBCs. The remaining granulocytes were washed twice with RPMI 1640 and resuspended in medium supplemented with 10% FBS and 2 mM glutamine (complete media). Mononuclear cells were isolated from the interphase after Ficoll-Paque density centrifugation and then washed twice with RPMI 1640 before being suspended in complete media. Granulocyte and mononuclear cell populations were determined to be >99% pure by differential cell counting after staining cells with Wright's stain. Cell viability was >99%, as determined by trypan blue exclusion.

ADCC. The lytic activity of neutrophils and mononuclear cells was determined by a ^{51}Cr-release assay using a neuroblastoma cell line, NMB7, as target cells (1×10^6) that were labeled with 0.2 mCi of $Na_2{}^{51}CrO_4$ for 4 h at 37°C. After

three washes with RPMI 1640, target cells were resuspended in complete media at 2×10^4 cells/ml, and 100 μl of this cell suspension were added to each well of a round-bottomed, 96-well plate containing either Ch14.18, Ch14.18 and GM-CSF, or Ch14.18-GM-CSF fusion protein and effector cells at E:T ratios of 100, 50, 25, and 10. Ch14.18 and GM-CSF were added to wells at concentrations matching those of the fusion protein on a molar basis. Final Ch14.18 concentrations were 0.05, 0.1, 0.5, and 1.0 μg/ml, and those of GM-CSF were 13, 26, 130, and 260 ng/ml, respectively. All conditions were performed in triplicate, and cells were incubated for 4 or 18 h at 37°C for granulocytes and mononuclear cells, respectively. Target cell lysis was determined by counting 100 μl of supernatant in a gamma scintillation counter after the collection of intact cells by centrifugation. Total and spontaneous lysis were determined by incubation of target cells in 0.2% SDS or in complete medium, respectively. The percentage of lysis was calculated by the formula:

$$\text{Lysis (\%)} = \frac{\text{Release in sample} - \text{spontaneous release}}{\text{Total lysis release} - \text{spontaneous release}} \times 100$$

The percentage of lysis is expressed by LU that were determined by using the exponential fit equation described by Pross *et al.* (22). One lytic unit is defined as the number of effector cells required to obtain 20% lysis of target cells.

CDC. NMB-7 cells were labeled with $Na_2{}^{51}CrO_4$ as described above for the ADCC assay. Labeled cells were plated at 2000 cells/well in a round-bottomed, 96-well plate containing increasing concentrations of either Ch14.18, Ch14.18 plus GM-CSF, or the Ch14.18-GM-CSF fusion protein and then incubated at 37°C for 1 h. The concentrations of Ch14.18 were matched to those of the fusion protein on a molar basis. Human sera from two normal donors were diluted 2- and 10-fold with RPMI 1640, and 100 μl were added to the wells containing target cells and incubated for 1.5 h at 37°C. Heat-inactivated serum was used as a control to ensure measurement of complement-specific lysis. Specific target cell lysis was determined as described above. Antibody-mediated CDC was determined by subtracting the percentage of tumor cell lysis attributable to complement alone.

RESULTS

ADCC. The binding of the Ch14.18-GM-CSF fusion protein to the target antigen G_{D2} was compared with that of Ch14.18 in a previous study (19). The results of an ELISA assay established that there was no significant difference in the binding of the Ch14.18-GM-CSF fusion protein and Ch14.18 to G_{D2}. To determine whether the immune-modulatory effect of GM-CSF and the biological activities of Ch14.18 are preserved in the Ch14.18-GM-CSF fusion protein, we performed ADCC assays using as effector cells granulocytes isolated from eight neuroblastoma patients and target cells from the neuroblastoma cell line NMB7. In two representative experiments (Fig. 1), the capacity of the Ch14.18-GM-CSF fusion protein to mediate tumor cell lysis by granulocytes was equivalent to that of the mixture of Ch14.18 and GM-CSF at all concentrations tested. Antibody-dependent tumor cell lysis peaked at 0.5–1 μg/ml of Ch14.18 with a GM-CSF concentration of 130–260 ng/ml.

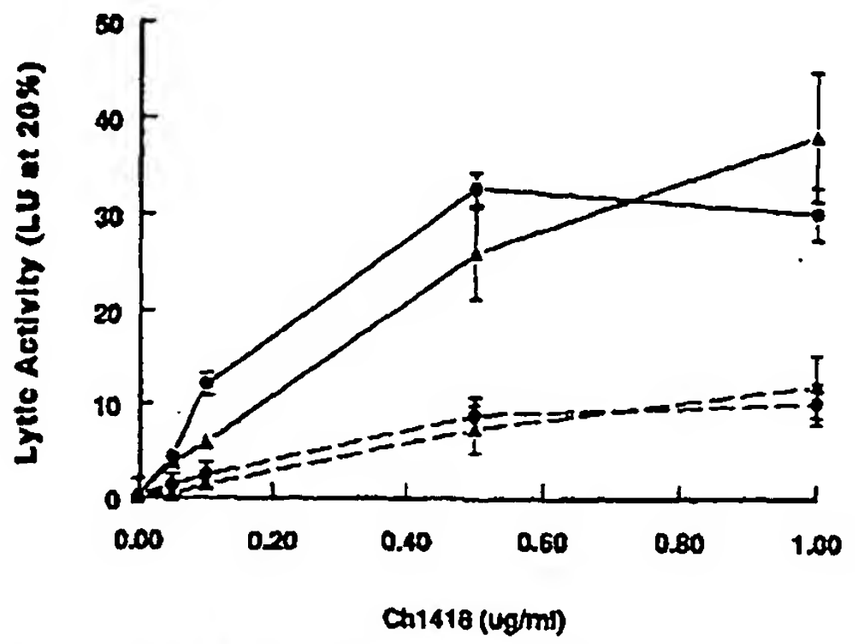

Fig. 1 Stimulation by GM-CSF of antibody-mediated lysis of neuroblastoma cells by granulocytes. Granulocytes isolated from peripheral blood of neuroblastoma patients were used as effector cells against NMB7 tumor cells in the presence of increasing concentrations of Ch14.18 plus GM-CSF (●) or Ch14.18-GM-CSF fusion protein (▲) in a 4-h ^{51}Cr-release assay as described in "Materials and Methods." The concentrations of free Ch14.18 and GM-CSF were matched on a molar basis to those present in the fusion protein. ———, patient 1006; —, patient U13. At least three independent ADCC experiments were performed for each of these two patients. Lytic activity of granulocytes alone was 0.5 ± 0.5 LU and 0 ± 0 LU for patients U13 and 1006, respectively. Specific lysis of target cells by up to 1109 g/ml of Ch14.18 or Ch14.18-GM-CSF alone was 1.0 ± 1.4%. *Bars*, SD.

Results of ADCC obtained with granulocytes from eight neuroblastoma patients are summarized in Table 1. The lytic activity mediated by Ch14.18 varied from patient to patient, but the addition of GM-CSF augmented tumor cell lysis mediated by Ch14.18 in six of eight samples, as observed by us previously (7). Importantly, tumor cell lysis mediated by the Ch14.18-GM-CSF fusion protein was essentially the same as that achieved by equivalent amounts of Ch14.18 plus GM-CSF in all but one patient (P1015). Furthermore, fusion protein-mediated tumor cell lysis was equivalent to that mediated by a mixture of Ch14.18 and GM-CSF, with granulocytes isolated from two different sources from patient U13, *i.e.*, peripheral blood and bone marrow. In addition, ADCC activity of peripheral blood mononuclear cells obtained from the same neuroblastoma patient (U13) on two occasions was the same with the fusion protein or a mixture of mAb Ch14.18 and GM-CSF at equivalent concentrations (Table 1). However, GM-CSF, either as part of the Ch14.18-GM-CSF fusion protein or as a separate entity, did not augment ADCC of peripheral blood mononuclear cells to the same extent as granulocytes of some patients (Table 1). Taken together, our results demonstrate that the fusion of GM-CSF to Ch14.18 does not alter the biological function of Ch14.18 in mediating ADCC of granulocytes and mononuclear cells nor affect the ability of GM-CSF to stimulate ADCC activity of these effector cells *in vitro*.

CDC. To determine whether the Ch14.18-GM-CSF fusion protein is able to direct CDC and whether this ability compares with that of mAb Ch14.18 alone, we performed CDC assays using sera from two normal donors as the complement source and cultured NMB7 human neuroblastoma cells as target.

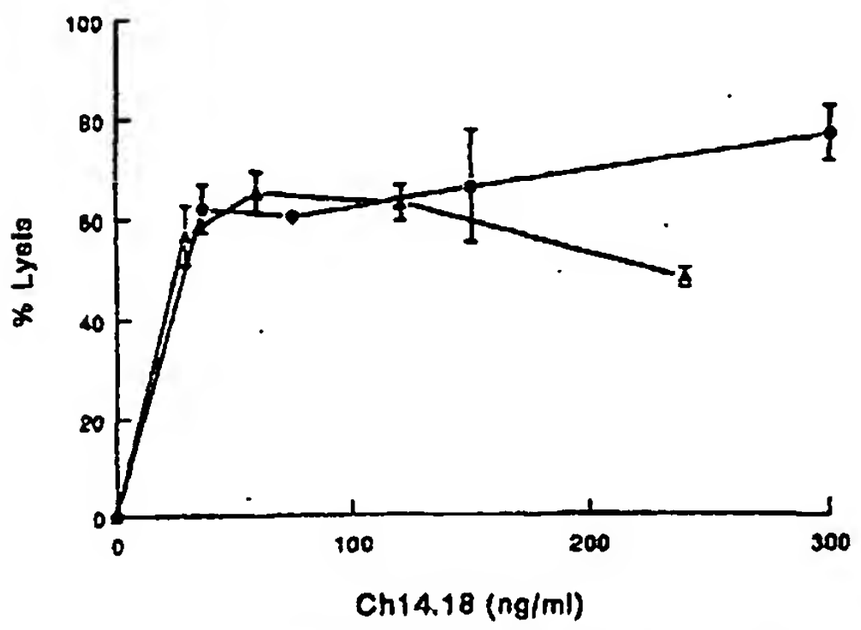

Fig. 2 Complement-dependent lysis of neuroblastoma cells. Serum from a normal donor diluted 2-fold was used as the complement source against NMB7 tumor cells in a 1.5-h ^{51}Cr-release assay after a 1-h preincubation at 37°C of NMB7 cells with increasing concentrations of Ch14.18 alone (●) or the Ch14.18-GM-CSF fusion protein (▲). The CDC assay was performed three times using sera from two different donors. Total lysis, 846 ± 113 cpm; spontaneous lysis, 75 ± 17 cpm; lysis by human complement alone, 5 ± 8 cpm. Bars, SD.

The results of a representative experiment among three experiments shown in Fig. 2 indicated that complement-dependent lysis was similar when mediated by either the Ch14.18-GM-CSF fusion protein or mAb Ch14.18. Interestingly, however, in two of three experiments, at concentrations of Ch14.18 >0.2 μg/ml, the fusion protein appeared to be somewhat less effective than Ch14.18 alone in directing complement-dependent lysis.

DISCUSSION

The Ch14.18-GM-CSF fusion protein was reported previously to bind G_{D2} to a similar extent as mAb Ch14.18 (19). We now demonstrate for the first time that this same fusion protein is capable of mediating ADCC against human neuroblastoma cells by granulocytes and mononuclear cells of neuroblastoma patients. Furthermore, the Ch14.18 moiety of the Ch14.18-GM-CSF fusion protein was equally effective as equivalent amounts of mAb Ch14.18 in mediating ADCC, and the GM-CSF moiety of this fusion protein was as effective as GM-CSF in enhancing ADCC mediated by granulocytes and mononuclear cells, when the mAb Ch14.18 and GM-CSF concentrations were matched on a molar basis. Previous *in vitro* studies with Ch14.18-IL-2 fusion protein (21) or a mAb-TNF-α conjugate (23) demonstrated that these were more effective in mediating ADCC compared with cytokine alone (21, 23) or mAb alone (21). A more meaningful comparison of ADCC activities is to compare those achieved with either the fusion protein or the combination of mAb and cytokine at equivalent concentrations. As observed previously by us (7, 15, 18), GM-CSF generally enhanced Ch14.18-mediated ADCC by granulocytes; however, now we demonstrated that this activity of GM-CSF was not diminished when fused to Ch14.18. The Ch14.18-GM-CSF fusion protein performed as well as Ch14.18 alone in directing CDC. However, at concentrations of Ch14.18 generally >0.2 μg/ml, the fusion protein appeared to be somewhat less effective in directing CDC than free Ch14.18. It is possible that at high concentrations, the structural features of the fusion protein may impose some steric hindrance to complement fixation *in vitro*.

Functions of immune effector cells are usually suppressed in most cancer patients, and many chemotherapeutic drugs induce immunosuppression and neutropenia (24, 25). Consistent with this observation, we found that granulocytes from six of eight neuroblastoma patients mediated very low ADCC with mAb Ch14.18 alone, yet in all but one case, GM-CSF enhanced granulocyte and mononuclear cell ADCC. The ability of GM-CSF to increase the production of granulocytes and mononuclear cells as well as to enhance their cytotoxic activities against tumor cells is well documented (11–16). In addition, GM-CSF can also affect the migration of granulocytes (7, 26), resulting in their increased accumulation at tumor sites (27). In view of the effects of GM-CSF on these effector cells, particularly granulocytes, the use of a Ch14.18-GM-CSF fusion protein in the treatment of neuroblastoma would be of considerable interest, especially because our present study indicates that the Ch14.18-GM-CSF fusion protein is equally effective in mediating ADCC *in vitro* as are mixtures of Ch14.18 and GM-CSF at equivalent concentrations. More importantly, on the basis of our *in vivo* studies with Ch14.18-IL-2 (28, 29), the Ch14.18-GM-CSF fusion protein would be expected to target GM-CSF and thereby direct granulocytes and mononuclear cells to the tumor microenvironment far more effectively than a combination of Ch14.18 and GM-CSF. In this regard, in addition to studies with Ch14.18-IL-2, earlier studies demonstrated that mAb-cytokine conjugates administered to mice could target cytokines to tumor sites more effectively than the administration of free cytokine (30). Furthermore, the fusion of GM-CSF to Ch14.18 would not only increase the half-life of GM-CSF but would also avoid or minimize toxicities of GM-CSF, as was observed with a bispecific antibody targeting TNF-α to tumor sites (31). A HAMA or an anti-GM-CSF response may occur against the Ch14.18-GM-CSF fusion protein. However, on the basis of results of *in vivo* studies (27, 28) obtained with a similar fusion protein, Ch14.18-IL-2, it does not appear that a HAMA or anti-GM-CSF response would occur or occur to any significant level to affect the antitumor effect of the Ch14.18-GM-CSF fusion protein. The results of the studies on the Ch14.18-IL-2 fusion protein demonstrated that the fusion protein was much more effective than the mixture of Ch14.18 and IL-2 in suppressing the growth of disseminated metastases of neuroblastoma to bone marrow and liver. Furthermore, the occurrence of a HAMA response may actually be beneficial because of the generation of an anti-id response. Recent studies have shown that generating an anti-id response was effective in obtaining an antitumor response (32, 33).Taken together, our results lend strong support for conducting clinical trials with a Ch14.18-GM-CSF fusion protein in neuroblastoma patients.

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3

ARTICLES

IL2–Ricin Fusion Toxin Is Selectively Cytotoxic *in Vitro* to IL2 Receptor-Bearing Tumor Cells

Arthur Frankel,*,† Edward Tagge,‡ John Chandler,‡ Chris Burbage,† Greg Hancock,† Joseph Vesely,§ and Mark Willingham§

Departments of Medicine, Surgery, and Pathology, Medical University of South Carolina, Charleston, South Carolina 29425. Received April 12, 1995®

Fusion toxins consist of peptide ligands linked through amide bonds to polypeptide toxins. The ligand directs the molecule to the surface of target cells and the toxin enters the cytosol and induces cell death. Ricin toxin is an excellent candidate for use in fusion toxins because of its extreme potency, the extensive knowledge of its atomic structure, and the years of experience with RTA chemical conjugates in clinical trials. We synthesized a baculovirus transfer vector with the polyhedrin promoter followed sequentially from the 5′ end with DNA encoding the gp67A leader sequence, the tripeptide ADP, IL2, another ADP tripeptide, and RTB. Recombinant baculovirus was generated in Sf9 insect cells and used to infect Sf9 cells. Recombinant IL2-RTB protein was recovered at high yields from day 5 insect cell supernatants, partially purified by affinity chromatography, and characterized. The recombinant product was soluble and immunoreactive with antibodies to RTB and IL2, bound asialofetuin and lactose, and reassociated with RTA. In the presence of lactose to block galactose-binding sites on RTB, the IL2–RTB–RTA heterodimer was selectively cytotoxic to IL2 receptor, bearing cells. Specific cytotoxicity could be blocked with IL2. Thus, we report a novel targeted plant toxin fusion protein with full biological activity.

INTRODUCTION

Ricin toxin, the 65 kDa heterodimeric glycoprotein from castor bean seeds, consists of a lectin B chain (RTB)[1] disulfide linked to an enzymatic A chain (RTA) (*1*). Ricin intoxication of mammalian cells involves sequentially (a) RTB binding to β-galactosyl pyranoside groups on cell surface glycoproteins (*2*), (b) internalization by endocytosis (*3*), (c) transfer to the TR Golgi (*4*), (d) routing to a critical organelle, possibly the endoplasmic reticulum (*5*), (e) disulfide bond reduction with release of RTA (*6*), (f) translocation of RTA to the cytosol, and (g) catalytic inactivation of protein synthesis by hydrolysis and release of an adenine base from the elongation factor binding site of 26S rRNA in the 60S ribosomal subunit (*7*). A single molecule of ricin introduced into a cell can lead to cell death (*8*).

Because of this extreme potency, a number of groups have attached RTA or modified ricin molecules to new ligands to achieve selective cell killing *in vitro* and *in vivo* and then used these immunotoxins systemically in patients with refractory neoplasms. The Fab′ fragment of a murine monoclonal anti-CD22 antibody was coupled to chemically deglycosylated RTA and administered intravenously to patients with B-cell lymphomas (*9*). While 50% of patients with antigen on the tumor cell surfaces showed a partial response, significant dose-limiting toxicity to vascular endothelium was observed. The small size (80 000 Mr) of the conjugate may have facilitated tumor penetration, but no studies of drug distribution were done. Mouse antibody to CD22 was coupled to chemically deglycosylated RTA and administered to lymphoma patients (*10*). Both partial and complete responses were observed, but again vascular leak syndrome was the dose-limiting toxicity. Higher peak concentration of immunotoxin in the serum, longer $T_{1/2}$, and larger AUC correlated with vascular injury. Murine monoclonal antibody to CD5 ws thiolated and coupled to RTA and administered to patients with chronic lymphocytic leukemia (*11*). At doses up to 16 mg/m², no immunotoxin could be demonstrated at extravascular sites. Mouse antibody to a 55 kDa epithelial cell surface glycoprotein was derivatized and conjugated to recombinant RTA and infused into patients with metastatic breast carcinoma (*12*). Four out of five patients developed anti-mouse Ig and anti-RTA antibodies. Ricin toxin was chemically blocked with an affinity ligand and cross-linked to an anti-CD19 monoclonal antibody (*13*). After administration to patients with lymphoma, different preparations of immunotoxin produced different degrees of hepatocyte damage. Further analysis revealed product heterogeneity with the more toxic species having one to two affinity ligands per ricin and the less toxic species with three affinity ligands per ricin. Thus, pharmacologic properties of drug heterogeneity, poor drug penetration, normal tissue toxicity, and immunogenicity have reduced the therapeutic index (ratio of dose producing toxicity/dose producing clinical efficacy) in clinical trials with RTA or blocked ricin immunotoxins. Methods have been

* Address correspondence to this author at the following address: Hollings Cancer Center Rm 306, MUSC, 171 Ashley Ave., Charleston, SC 29425. Tel: 803-792-1450. Fax: 803-792-3200.
† Department of Medicine.
‡ Department of Surgery.
§ Department of Pathology.
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[1] Abbreviations: IL2, interleukin-2; RTB, ricin toxin B chain; RTA, ricin toxin A chain; IL2–RTB, fusion protein of interleukin-2 and ricin B chain.

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sought to improve the pharmacologic properties of these protein therapeutics.

One approach has been to genetically engineer the toxin and ligand into a single well-defined molecule. Normal cell binding portions of diphtheria toxin and *Pseudomonas* exotoxin have been deleted and replaced with growth factors and single chain Fv's (*14*, *15*). Clinical trials with TGFα-PE40 and DAB_{389}IL2 bacterial fusion toxins have shown excellent tolerance (minimal side effects or toxicities) and significant clinical activity (*16*, *17*). A fusion protein consisting of basic fibroblast growth factor and the ribosome-inactivating protein saporin (FGF-SAP) has been expressed in *E. coli* (*18*). FGF-SAP demonstrated potent specific cell cytotoxicity ($ID_{50} = 5 \times 10^{-11}$ M) and inhibited growth of B16 melanomas both in subcutaneous implants and lung metastases in mice. Surprisingly, few studies have been done directly comparing fusion toxins with chemical conjugates. Lappi reported no difference in vitro or in vivo between FGF-SAP linked by a cross-linker or by an amide bond (*18*). Kreitman compared anti-Tac-PE with the single chain fusion toxin anti-Tac(Fv)-PE40 (*19*, *20*). In vitro and in vivo, the fusion toxin was approximately 1 log more active. An additional advantage of the fusion toxin was the reagent homogeneity.

Attempts have been undertaken to produce genetically engineered ricin or RTA fusion proteins, in part because of the extensive clinical experience with RTA and blocked ricin immunotoxins showing safety in patients (*9*–*13*) and, in part, due to the potential use of ricin fusion toxins in patients who have developed resistance to diphtheria toxin or *Pseudomonas* exotoxin fusion proteins. RTA inactivates protein synthesis by specifically depurinating a conserved adenosine in the 60S ribosomal subunit, while diphtheria toxin and *Pseudomonas* exotoxin act by ADP-ribosylating EF-2 (*7*). Thus, malignant cells are unlikely to show cross-resistance to both plant and bacterial toxins. Further, the amino acid sequence and three-dimensional structure of ricin is distinct (*21*) from the diphtheria toxin and *Pseudomonas* exotoxin, and thus, antibodies to one toxin do not react with the other toxins (unpublished observations). An RTA–diphtheria toxin loop–Staphylococcal protein A fusion protein was expressed in *E. coli*, enzymatically cleaved with trypsin, mixed with antibody, and exposed to antigen positive cells (*22*). Selective cytotoxicity was demonstrated. However, the fusion toxin antibody conjugate had unfavorable properties. The disulfide loop was exposed on the surface of the conjugate and readily reduced. The RTA–diphtheria toxin–protein A–immunoglobulin conjugate was very large (>200 000 Da). The conjugate was heterogeneous due to varying sites of protein A-immunoglobulin binding. Subsequently, tripartate fusion proteins were produced in *E. coli* with IL2–diphtheria toxin loop–RTA or IL2–factor Xa recognition sequence–RTA (*23*). Proteolytic cleavage with trypsin or factor Xa released the IL2 ligand and toxin without recovery of disulfide-linked product. Uncleaved chimeras showed no cytotoxicity to IL2 receptor-bearing cells. Finally, a factor Xa-specific site was introduced into the linker sequence of proricin and the modified proricin expressed in *Xenopus* oocytes (*24*). Although recombinant mutant proricin was produced, yields were in the nanogram range and protease sensitivity was low. Further, no fusion proteins with IL2 binding specificities were made.

We chose an alternative approach for genetic engineering of ricin. Previous studies document the need for an accessible disulfide bridge between ligand and RTA (*6*, *22*). Consequently, we chose to produce recombinant RTB fusion proteins with novel ligands and reassociate the molecule with RTA to recreate the natural disulfide bridge between RTA and RTB. The ligand was attached to the N-terminus of RTB based on our previous experience with an oligohistidine tag and the X-ray crystallographic structure of ricin (*21*, *25*). In the present study, we synthesized DNA encoding the GP67A leader peptide, IL2, and RTB. Recombinant protein was expressed and secreted from insect cells. The fusion molecule was purified, reassociated with plant RTA, and tested for selective cytotoxicity to IL2 receptor positive cells in the presence of lactose.

EXPERIMENTAL PROCEDURES

Materials. Restriction endonucleases and T4 ligase were obtained from Promega (Madison, WI). [^{32}P]dCTP, [^{35}S]dATP, [^{3}H]leucine were obtained from Amersham (Arlington Heights, IL). Rabbit antiricin antibody, alkaline phosphatase conjugated goat anti-(rabbit IgG), alkaline phosphatase conjugated goat anti-(mouse IgG), asialofetuin, α-lactose, and other chemicals were from Sigma (St. Louis, MO). EX-CELL400 medium was obtained from JRH Scientific (Lexena, KS). Sf9 insect cells, TMNFH medium, BaculoGold DNA, and pAcGP67A transfer fector were from PharMingen (San Diego, CA). Prep-A-Gene DNA and plasmid purification matrices, low molecular weight prestained protein standardards, nitrocellulose paper, and other reagents for protein analysis were obtained from BioRad (Hercules, CA). The Sequenase kit for dideoxy sequencing was obtained from USB (Cleveland, OH). The Random Primer labeling kit was obtained from Stratagene (La Jolla, CA). Purified P2, P8, and P10 murine monoclonal antibodies to RTB and purified αBR12 murine monoclonal antibody to RTA were gifts of Dr. Walter Blattler, ImmunoGen (Cambridge, MA). RPMI1640 media, leucine-free RPMI1640, penicillin, streptomycin, Dulbecco's PBS, fetal bovine serum, and dialyzed fetal bovine serum were obtained from GIBCO BRL (Grand Island, NY). 3M Emphaze Biosupport medium AB1 azlactone functionality bis-acrylamide and lactosyl acrylamide were obtained from Pierce (Rockford, IL). The alkaline phosphatase Vectastain kit for Western blots was obtained from Vector Laboratories (Burlingame, CA). EIA plates and round-bottomed and flat-bottomed 96-well plates were from Costar (Cambridge, MA). Plant RTB, ricin, and RTA were obtained from Inland Laboratories (Austin, TX).

Construction of Plasmid. pDW27 plasmid containing DAB_{389}IL2 DNA was a gift of Dr. John Murphy (Boston University) (*27*). PCR was performed with pDW27 plasmid encoding DAB_{389}IL2 and the 5′ oligonucleotide 5′-GCAGCATCAGGATCCCGCACCTACTTCTA-GCTCT-3′, which introduces a BamHI site followed by a CCC proline codon followed by DNA encoding the first six codons of IL2. The 3′ oligonucleotide was 5′-AGCTGCAGATGGAT-CGCGGTCAGGGTAGAGATGAT-3′, which contains the last six codons of IL2 followed by a GC and a BamHI site. The PCR product, which provided an IL2 DNA BamHI cassette maintaining the proper reading frame at both ends with the GP67A leader and RTB, was purified on a silica matrix (Prep-A-Gene, BioRad), digested with BamHI, and subcloned in BamHI restricted pAcGP67A-RTB plasmid (*24*). The final vector was double-stranded dideoxy sequenced by the Sanger method with Sequenase reagents (USB). One liter cultures of transformed *E. coli* were subjected to alkaline lysis, and the plasmid was purified by cesium chloride density gradient centrifugation.

Expression of Fusion Toxin. A 2×10^6 Sf9 sample of *S. frugiperda* ovarian cells maintained in TMNFH medium supplemented with 10% fetal calf serum was

cotransfected with pAcGP67A–IL2–RTB DNA (4 μg) and 0.5 μg of BaculoGold AcNPV DNA following the recommendations of the supplier (PharMingen). At 5 days post-transfection, medium was centrifuged and the supernatant tested in a limiting dilution assay with Sf9 cells and dot blots with random primer ^{32}P-dCTP labeled RTB DNA (Stratagene Prime-It kit) as previously described (*28*). Positive wells were identified and supernatants reassayed by limiting dilution until all wells up to 10^{-7} dilution were positive. Two rounds of selection were required. Recombinant virus in the supernatant was then amplified by infecting Sf9 cells at a multiplicity of infection (moi) of 0.1, followed by collection of day 7 supernatants. Recombinant baculovirus was then used to infect 2 × 10^8 Sf9 cells at an moi of 5 in 150 mL of EX-CELL400 medium (JRH Scientific) with 50 mM α-lactose in spinner flasks. Media supernatants containing IL2–RTB were collected day 6 postinfection.

Purification of IL2–RTB. Media supernatants were adjusted to 0.01% sodium azide and maintained through all purification steps at 4 °C. The supernatants were concentrated 15-fold by vacuum dialysis, centrifuged at 3000*g* for 10 min to remove precipitate, dialyzed against 50 mM NaCl, 25 mM Tris pH 8, 1 mM EDTA, 0.01% sodium azide, and 25 mM α-lactose (NTEAL), ultracentrifuged at 100000*g* for 1 h, and bound and eluted from a P2 monoclonal antibody–acrylamide matrix as previously described (*28*). P2 is an anti-RTB monoclonal antibody. The affinity matrix was prepared using Ultralink azalactone functionality bis-acrylamide following the recommendations of the manufacturer. Recombinant protein was adsorbed to the column in NTEAL, washed with 500 mM NaCl, 25 mM Tris pH 8, 1 mM EDTA, 0.1% Tween 20, 0.01% sodium azide, and 25 mM α-lactose, and eluted with 0.1 M triethylamine hydrochloride pH 11. The eluant was neutralized with 1/10 volume 1 M sodium phosphate pH 4.25 and stored at −20 °C until assayed. Four preparations were made.

Molecular Weight Determination. Aliquots of IL2-RTB, recombinant RTB, plant RTB (Inland Laboratories), and prestained low molecular weight standards (BioRad) were run on a reducing 15% SDS/PAGE, stained with Coomassie Blue R-250, dried between cellophane sheets, and scanned on a IBAS 2000 automatic image analysis system (Kontron, Germany).

Immunological Characterization. Aliquots of IL2-RTB, bacterial IL2 (Chiron), wild-type recombinant RTB, plant RTB, and prestained low molecular weight protein standards were run on a reducing 15% SDS–PAGE, transferred to nitrocellulose, blocked with 10% Carnations nonfat dry milk/0.1% BSA/0.1% Tween 20, washed with PBS plus 0.05% Tween 20, reacted with either 1:400 rabbit antibody to ricin (Sigma) or 1:100 mouse monoclonal antibody to IL2 (Genzyme), rewashed, incubated with alkaline phosphatase conjugated goat anti-(rabbit IgG) or anti-(mouse IgG) (Sigma), washed again, and developed with the Vectastain alkaline phosphatase kit (Vector Laboratories).

Costar EIA microtiter wells were coated with 100 μL of 5 μg/mL of monoclonal antibody P2, P8, or P10 reactive with RTB (gifts of Dr. Walter Blattler, ImmunoGen) or monoclonal antibody to IL2 (Genzyme), washed with PBS plus 0.1% Tween 20, blocked with 3% BSA, rewashed, and incubated with samples of IL-2-RTB or plant RTB, rewashed, reacted with 1:400 rabbit antibody to ricin, washed again, incubated with 1:5000 alkaline phosphatase conjugated goat anti-(rabbit IgG), rewashed, developed with 1 mg/mL of *p*-nitrophenyl phosphate in diethanolamine buffer pH 9.6, and read on a BioRad 450 Microplate reader at 405 nm.

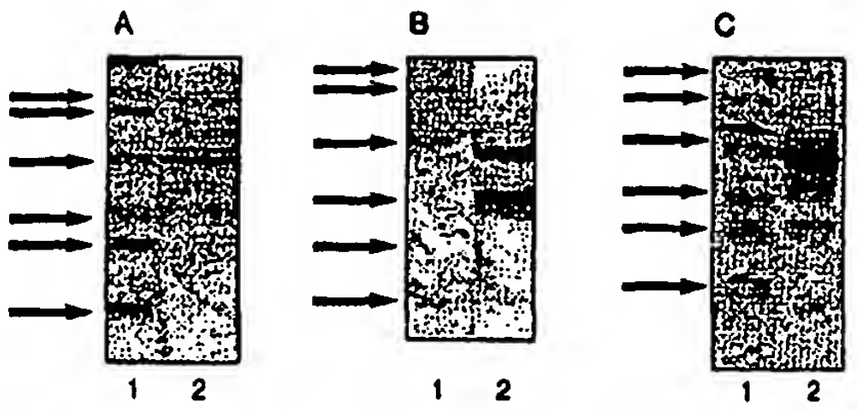

Figure 1. Fifteen percent reducing SDS–PAGE of IL2–RTB. (A) Coomassie stained. Lane 1: low molecular weight prestained BioRad protein standards as marked by arrows and are 20 000, 28 500, 34 400, 52 ,000, 86 000, and 112 000 daltons; lane 2: IL2–RTB. (B) Immunoblot using rabbit anti-ricin antibody. Lane 1: low molecular weight protein standards marked by arrows as above. Lane 2: IL2–RTB. (C) Immunoblot using mouse anti-IL2 antibody. Lane 1: low molecular weight standards. Lane 2: IL2–RTB.

Measurement of Lectin Activity. Asialofetuin (1 μg/mL) was bound to Costar EIA plate wells, and an ELISA was performed as previously detailed with samples of IL2–RTB and plant RTB (*28*). Samples of freshly reduced IL2–RTB were diluted in 50 mM NaCl/25 mM Tris pH 8/1 mM EDTA/0.01% sodium azide (NTEA), loaded on a lactosyl acrylamide matrix (Pierce), and washed with NTEA, and the lactose binding protein was eluted with NTEA plus 50 mM lactose. Fractions were assayed for RTB immunoreactive material using the P2 antibody ELISA described above.

Formation of Recombinant Heterodimer. Thirty μg of IL2–RTB was mixed with 90 μg of plant RTA in a total volume of 1 mL of PBS and then shaken overnight at room temperature. The reaction mixture was then analyzed by a ricin ELISA previously described (*25*). Reassociated mixtures were also analyzed by nonreducing SDS–PAGE followed by immunoblots with P10 anti-RTB monoclonal antibody and monoclonal antibody to IL2. Densitometric scanning with the automatic image analysis system was done to quantify the shift of immunoreactive material from 50 to 80 kDa.

Cytotoxicity to Mammalian Cells. Measurement of protein synthesis inhibition by ricin, IL2–RTB–RTA, and DAB_{389}IL2 (*14*) in cultured cells was done as previously described using HUT102 human T leukemia cells bearing the high affinity IL2 receptor, CEM human T leukemia cells bearing the intermediate affinity IL2 receptor, and OVCAR3 human ovarian carcinoma cells lacking the IL2 receptor. All three cell lines were obtained from the American Type Culture Collection (Rockville, MD). HUT102 cells intermittently release HTLV-1 and should be handled with care. All assays were performed in triplicate. In some experiments duplicate samples were incubated in the presence of 20 μg/mL of IL2 (Chiron). The ID_{50} was the concentration of protein which inhibited protein synthesis by 50% compared with control.

A total of 1.5 × 10^4 HUT102 cells were placed in sterile Eppendorf tubes at 4 °C in 100 μL of leucine-poor RPMI1640 + 10% dialyzed fetal calf serum + 60 mM α-lactose with or without 20 μg/mL of IL2. Dilutions of IL2–ricin and ricin at varying concentrations were added in identical medium with or without IL2 and incubated at 4 °C for 30 min. Cells were pelleted at 2000*g* for 5 min, washed once with leucine-poor RPMI1640 + 10% dialyzed fetal calf serum + 60 mM α-lactose, resuspended in 150 μL of the same medium, and incubated at 37 °C in 5% CO_2 for 24 h. [^{3}H]leucine was added as above, and 4 h later cells were harvested with the PhD cell harvester

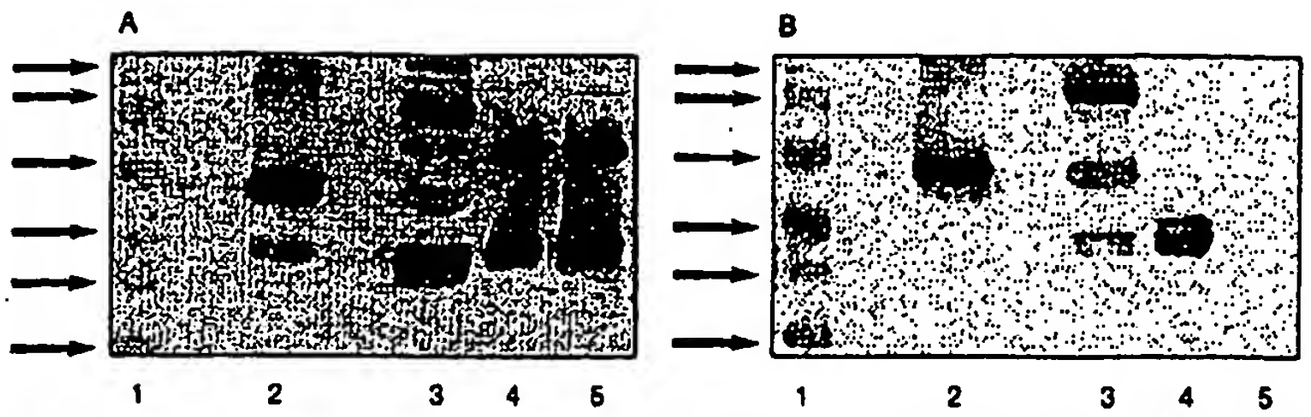

Figure 2. Fifteen percent nonreducing SDS–PAGE of IL2–RTB–RTA. (A) Immunoblot using mouse monoclonal antibody P10 to RTB. Lane 1: low molecular weight standards. Lane 2: IL2–RTB. Lane 3: IL2–RTB–RTA. Lane 4: RTA–plant RTB. Lane 5: RTA–recombinant RTB. (B) Immunoblot using mouse monoclonal antibody to IL2. Lane 1: low molecular weight standards. Lane 2: IL2–RTB. Lane 3: IL2–RTB–RTA. Lane 4: RTA–plant RTB. Lane 5: RTA–recombinant RTB. Densitometry of gel B confirmed 85% of the RTA immunoreactive material migrated at 80 kDa after reassociation.

and incorporated [^{3}H]leucine measured in a liquid scintillation counter. Blocking of selective cytotoxicity was estimated by comparing the ID_{50} of toxins in the presence or absence of IL2 + lactose. There was no purification step after heterodimer reassociation. The free RTA concentration at the highest concentration of heterodimer in the assay (5×10^{-7} M) was 10^{-7} M. On all three cell lines, the ID_{50} for free RTA was ($2–3 \times 10^{-6}$ M) (unpublished results). Thus, in the range of heterodimer ID_{50}'s ($10^{-10}–10^{-12}$ M), the free RTA concentration (2×10^{-11} M to 2×10^{-13} M) should not produce cytotoxicity.

RESULTS

Yield and purity of IL2–RTB. Material post-affinity chromatography was subjected to SDS–PAGE and Coomassie staining (Figure 1A). Over 80% of the protein migrated as a single band at 50 kDa. On the basis of absorbance at 280 nm (1 mg/mL produced an OD_{280} = 1.4) and densitometry, 1 mg/mL fusion protein was secreted per liter of insect cell culture. The major contaminants at 55, 60, and 90 kDa were not reactive with murine antibodies against RTA or IL2 and appeared in media of cells after lysis from wild-type AcNPV virus. They presumably represent insect cell derived proteins. Individual batches of IL2-RTB of about 150 μg were recovered from 150 mL of infected insect cell supernatants and were each adequate for all the studies undertaken.

Immunologic Cross-Reactivity. Immunoblots demonstrated reactivity with the same 52 kDa band using anti-RTB or anti-IL2 antibodies (Figure 1B,C). Interestingly, weaker bands of approximately 10–15% intensity relative to the 52 kDa band were observed at 35 kDa reactive with anti-RTB and 20 kDa reactive with anti-IL2 suggesting partial proteolysis occurred either intracellularly or in the medium. The site(s) of cleavage are unknown, but the size of the fragments and their specific reactivities with antibodies suggest the predominant site of cleavage is between the RTB and IL2 domain. This is not unusual for chimeras, as the secondary structure is likely to be least between domains.

Antibody ELISA's demonstrated similar reactivity of IL2–RTB and plant RTB with anti-RTB monoclonal antibodies. Relative to plant RTB, the monoclonal antibody P2 reacted 66% as well with IL2–RTB. P8 and P10 monoclonal antibodies reacted 100% as well with IL2-RTB molecules as with plant RTB. Anti-IL2 antibody reacted with at least 70% of the RTB immunocross-reactive molecules on a molar basis.

Lectin Activity. α-Lactose was coupled through the 6-hydroxyl groups of the glucosyl moiety to acrylamide, and binding of lectins was assessed. Plant RTB bound 100% to immobilized lactose, while 80% of recombinant wild-type RTB could be bound and eluted from lactosyl acrylamide. Thirty percent of IL2–RTB attached to the matrix and eluted with 50 mM α-lactose.

Table 1. Cell Cytotoxicity of Toxins to Various Cell Lines

protein	ID_{50} (M) HUT102	CEM	OVCAR3
ricin	$(5 \pm 2) \times 10^{-10}$	$(2 \pm 1) \times 10^{-10}$	$(5 \pm 3) \times 10^{-10}$
IL2–RTB–RTA	$(4 \pm 1) \times 10^{-12}$	$(2 \pm 1) \times 10^{-10}$	$(6 \pm 2) \times 10^{-10}$
$DAB_{389}IL2$	$(2 \pm 1) \times 10^{-12}$	$(5 \pm 3) \times 10^{-9}$	$>10^{-6}$

[a] 2×10^4 cells in 150 μL of leucine-free RPMI1640 plus 60 mM α-lactose were combined with dilutions of toxins for 24 h at 37 °C/5% CO_2. 1 μCi/well ^{3}H leucine in 50 μL of the same media was added for 4 h at 37 °C/5% CO_2. Cells were harvested with a PhD cell harvestor on glass fiber mats, dried, and counted in Econofluor in a LKB liquid scintillation counter. ID_{50} was the concentration of toxin reducing protein synthesis by 50%. Each assay perfomed three times. Standard deviations shown with mean.

Asialofetuin consists of the bovine serum protein fetuin from which the terminal sialic acids have been chemically removed, exposing galactosyl residues. Binding of IL2–RTB to asialofetuin adsorbed on microtiter wells provides an independent assay of galactose binding affinity. The asialofetuin ELISA demonstrated that IL2–RTB bound immobilized asialofetuin 59% as well as plant RTB and wild-type recombinant RTB. Both the lactose binding and asialofetuin binding assays suggest the fusion molecule retains much of the lectin activity of wild-type recombinant RTB. The slight reduction in binding affinity may be secondary to steric hindrance by IL2 or misfolding affecting one or more sugar-combining subdomains. This residual lectin property is not desirable for an *in vivo* therapeutic molecule.

Reassociation with RTA. IL2–RTB provides a ligand function and coupling function for the fusion toxin, but the polypeptide must be linked to RTA for cytotoxicity. RTB has numerous amino acid residues which interact with RTA and promote both stabilization of the toxic heterodimer and protection of the intersubunit disulfide bridge (*21*). Provided the IL2 amino acid residues do not lead to misfolding or steric hindrance of the interface sidechains, reassociation of IL2–RTB with RTA should occur spontaneously at concentrations of 10^{-6} M or higher of each component (*26*). Under the reaction conditions (10^{-6} M, PBS, room temperature, room air), 50% reassociation was achieved with plant RTA and either plant or recombinant RTB. Under identical conditions, 60% reassociation of IL2–RTB occurred based on ricin ELISA and immunoblots with anti-RTB antibody (Figure 2).

Cell cytotoxicity. Cytotoxicities of recombinant proteins and plant ricin for different cell lines are shown in Table 1. IL2–RTB alone was nontoxic ($ID_{50} > 10^{-6}$ M) for all the cell lines tested. Ricin and IL2–ricin showed some toxicity ($ID_{50} = 1–6 \times 10^{-10}$ M) to IL2 receptor

negative cell lines in the presence of 60 mM α-lactose (Figure 3B,C). This residual toxicity is due to the presence of lectin binding sites on ricin and IL2–ricin that are incompletely blocked by 60 mM α-lactose. In contrast, IL2–ricin but not ricin was much more toxic to the IL2 receptor-positive HUT102 cells ($ID_{50} = 4 \times 10^{-12}$ M) in the presence of lactose (Figure 3A). The *in vitro* therapeutic window with lactose in the media was 125-fold. The control fusion toxin, $DAB_{389}IL2$, also showed selective toxicity to IL2 receptor-bearing cells. Since $DAB_{389}IL2$ lacks residual normal cell binding domains, the toxicity to nonreceptor bearing cells was considerably less (ID_{50}'s = $(5-15) \times 10^{-9}$ M).

If the increased sensitivity of HUT102 cells to IL2–ricin in the presence of lactose is due to receptor-specific binding, we should be able to block binding in the presence of excess IL2. HUT102 cells were exposed to IL2 (20 μg/mL) and 60 mM lactose or lactose alone with varying concentrations of IL2–ricin or ricin for 30 min at 4 °C. After cells were washed and then incubated overnight at 37 °C/5% CO_2, cellular protein synthesis was measured by incorporation of [^{3}H]leucine. Each assay was repeated three times. The ID_{50} of ricin with standard deviation was $(3 \pm 2) \times 10^{-10}$ M in the presence of IL2 and $(6 \pm 3) \times 10^{-10}$ M in the absence of IL2. In contrast, the ID_{50} of IL2–ricin with standard deveiations was $(5 \pm 4) \times 10^{-12}$ M without IL2 and $(3 \pm 2) \times 10^{-10}$ M in the presence of excess IL2. The slightly lower specific toxicity of IL2–ricin under these conditions was due to the shortened exposure time.

DISCUSSION

We report the construction and expression of a novel ricin fusion protein in significant yields. The baculovirus expression system has been previously used to independently express human IL2 and ricin toxin B chain (*28*, *29*). We now describe the expression of the hybrid protein, IL2–RTB. Other chimeric eukaryotic proteins have been successfully expressed in insect cells using recombinant baculovirus including a fusion of the F and HN glycoproteins of human parainfluenza virus type 3 and a single chain monoclonal antibody composed of the variable domain of the light chain connected through AGQGSSV to the variable domain of the heavy chain (*30*, *31*). In each case, the fusion protein needed a signal sequence for segregation into the endoplasmic reticulum with subsequent proper folding, disulfide bond formation, and glycosylation. The folding of each portion of IL2–RTB was facilitated by attachment of IL2 to the N-terminus of RTB. The x-ray crystallographic three-dimensional structure of ricin shows the N-terminus of RTB participates to a small degree in folding of RTB and interaction with RTA (*21*). Our results extend our earlier observation that an oligohistidine tag attached to the N-terminus of RTB preserved folding for both the oligohistidine peptide and RTB (*25*). These observations provide the foundation for a general strategy of producing ricin fusion proteins by attachment of novel ligands to the RTB N-terminus.

Evidence in addition to secretion, solubility and yield that IL2-RTB had proper folding of both the IL2 and RTB domains was the similar immunological reactivity of soluble IL2–RTB, RTB, and IL2 with antibodies to IL2 and RTB. Anti-IL2 antibody bound IL2 and IL2–RTB comparably, and anti-RTB antibodies bound RTB and IL2–RTB approximately equally (see Results). TheELISA assay format permitted quantitative measurements of soluble proteins.

IL2–RTB retained most of the galactose-binding activity of plant and wild-type recombinant RTB. The X-ray

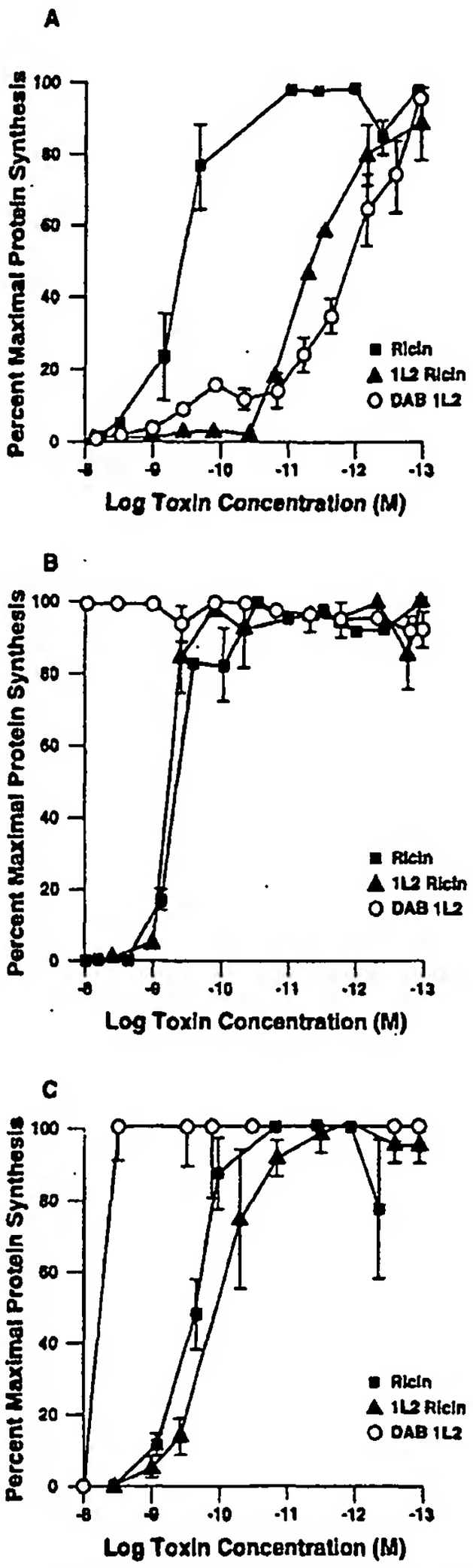

Figure 3. (A) HUT102 cell cytotoxicity measured by inhibition of protein synthesis using [^{3}H]leucine incorporation. See text for details of assay. Key: ■, ricin in the presence of 60 mM lactose; ▲, IL2–RTB–RTA in the presence of 60 mM lactose; O, $DAB_{389}IL2$ in the presence of 60 mM lactose. (B) Same as A except OVCAR3 cell cytotoxicity. (C) Same as A except CEM cell cytotoxicity. Standard deviations shown for single assay performed in triplicate. Standard deviation bars < 2% not shown. Each assay performed on three separate occasions, and standard deviations of ID_{50}'s shown in Table 1.

structure of ricin cocrystallized with lactose showed lectin sites in the 1α and 2γ subdomains remote from the N-terminus of RTB (*21*). Their independent behavior from the ligand will permit the systematic modification of sugar binding in recombinant ricin fusions. Previous work by Youle, Goldmacher, and Newton suggested some

residual lectin activity was critical for RTB enhancement of targeted ricin immunotoxin cytotoxicity (*4*, *32*, *33*).

Since the K_a of RTA–RTB association is 10^{-6} M^{-1} and our experiments employed concentrations of about 10^{-6} M, we expected 50% of IL2–RTB to reassociate with RTA (*26*). Our observation of 20% heterodimer formation suggests moderate efficiency of reassociation. Again, the X-ray structure shows the RTB amino acid residues interacting with RTA include RTB A1, D2, C4, F140, V141, F218, K219, N220, P260, and F262 (*22*). Thus, several residues are near the N-terminus and may affect reassociation, but the majority are distant from the surface N-terminal region. The recombinant heterodimers were stable at high dilutions, suggesting disulfide bond formation between RTA C259 and the free thiol of RTB C4 of IL2–RTB.

The IL2–RTB molecule alone was nontoxic to cells with or without the IL2 receptor and with or without lactose (see Results). Thus, no membrane lytic or other toxophore functions have been detected on the RTB moiety either alone or when targeted to cell surfaces with an alternate ligand. In contrast, IL2–RTB–RTA showed cytotoxicity in the absence of lactose indistinquishable from ricin. Thus, all the intoxication functions of ricin were present on the IL2–ricin fusion protein. In the presence of lactose, IL2–RTB–RTA showed selective and potent cytotoxicity to cells bearing the high affinity IL2 receptor (HUT102). The level of cytotoxicity to high affinity IL2 receptor containing cells was on the same order of magnitude as $DAB_{389}IL2$, a diphtheria toxin–IL2 fusion currently in clinical trials, and suggested the ricin-based genetically engineered immunotoxin was potent. Cells containing low levels of intermediate affinity receptor (CEM) or no receptor (OVCAR3) were no more sensitive to IL2–RTB–RTA than ricin alone in the presence of lactose. Future work will be focused on the partial removal of lectin function in the fusion toxin by site-specific mutagenesis. Such fusion toxins lacking significant normal tissue binding could then be studied in animal models of leukemia, lymphoma, or autoimmune diseases. We have prepared single-site and double-site RTB mutants. To date, significant residual cell binding and heterodimer cytotoxicity has been observed even with double-site mutants, suggesting three or more lectin sites on RTB (unpublished observations).

Competition experiments with excess ligand have been a frequently used method for confirming specificity of ligand–toxins (*14*, *15*). Initial efforts to block our IL2–toxins with excess IL2 resulted in complete blockage of $DAB_{389}IL2$ cytotoxicity but minimal reductions in IL2–RTB–RTA killing. We reasoned that the residual lectin binding of the chimeric protein may have been strengthened after interaction with the cell surface and that the multivalent binding accelerated internalization at 37 °C. When we reduced the temperature to 4 °C to impair internalization and shortened exposure time to 30 min, we were able to block cytotoxicity with IL2 plus lactose. Thus, we were able to confirm the extreme rapidity of ricin fusion protein intoxication of cells and establish IL2 receptor specificity of the molecule.

This report describes successful production of a ricin toxin fusion protein with full biological potency. The reagent was produced in sufficient amounts and purity for studies of cell intoxication pathways. In the future, modified forms of this fusion protein (with reduced RTB lectin character) may be useful for *in vitro* and *in vivo* purging of IL2 receptor bearing cells.

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IL2 fused to lectin-deficient ricin is toxic to human leukemia cells expressing the IL2 receptor

AE Frankel[1], T Fu[1], C Burbage[1], J Chandler[2], MC Willingham[3] and EP Tagge[2]

Departments of [1]Medicine, [2]Surgery and [3]Pathology, Medical University of South Carolina, Charleston, SC, USA

Interleukin-2 (IL2) fused to ricin B chain (RTB) with modifications of amino acid residues in each of three galactose-binding subdomains (1α, 1β and 2γ) was expressed in insect cells, purified by immunoaffinity chromatography and reassociated with ricin A chain (RTA). The fusion toxin-bound human leukemic cells with IL2 receptors and the binding was competed with IL2 but not asialofetuin. In contrast, binding was not observed with receptor negative human cell lines, and the fusion molecule very weakly bound asialofetuin ($K_d = 10^{-6}$M), indicating lectin-deficient RTB. The IL2–lectin-deficient RTB-RTA intoxicated IL2 receptor bearing cells as well as ricin or IL2–wild-type RTB-RTA. While ricin and IL2–wild-type RTB-RTA were equally toxic to receptor negative cell lines, the IL2–lectin-deficient RTB-RTA was two-two and one half logs less cytotoxic to these cell lines. The sensitivity of receptor-positive cells to the lectin-deficient fusion protein suggests that high avidity intracellular galactose binding may not be required for ricin intoxication, at least in the case of IL2 receptor-targeted molecules. Furthermore, the potent selective cytotoxicity of the fusion protein suggests that the IL2–lectin-deficient RTB-RTA and similar ricin fusion molecules directed against other leukemic cell surface receptors provide a novel class of fusion toxins for therapy of human leukemias.
Keywords: ricin; fusion toxin; IL2 receptor

Introduction

Many patients with hematopoietic malignancies have incomplete responses to chemoradiotherapy and die from progressive disease. Patients' leukemic blasts may develop multiple drug resistance phenotypes and normal tissue toxicities may limit dose escalation. Novel therapeutic modalities with minimal toxicities and no cross-resistance with current cytotoxic treatments are needed.

One such class of drugs are fusion toxins which are hybrid proteins composed of peptide ligands reactive with malignant cells (antibody fragments or cytokines) fused to polypeptide toxins (diphtheria toxin, Pseudomonas exotoxin or ricin). The toxin–ligand–receptor complex internalizes into intracellular compartments from which the catalytic domain of the toxin translocates to the cytosol and inactivates protein synthesis.

The target for several leukemia-directed fusion toxins has been the IL2 receptor (IL2R). IL2R is a heterotrimeric glycoprotein complex on the cell membrane with a 55 kDa α subunit, a 75 kDa β subunit and a 64 kDa γ subunit.[1] The only normal human tissues expressing IL2Rα and IL2Rβ are activated T cells, B cells, LGL cells and monocytes and some liver Kupffer cells, lung macrophages and skin Langerhans' cells. A variety of hematologic neoplasms may show high affinity IL2R expression including hairy cell leukemia, adult T cell leukemia, and a fraction of cutaneous T cell lymphomas and B cell chronic lymphocytic leukemias.[2]

Diphtheria toxin (DT) and Pseudomonas exotoxin (PE) have been fused to either IL2 or antibody Fv anti-IL2R peptides.[3–10] All reagents showed potent selective cytotoxicities *in vitro*, in some cases, *in vivo*.

Ricin-based fusion proteins are attractive candidates for development for several reasons. (1) The toxin inactivates cell protein synthesis by a mechanism independent of that used by DT or PE. The RNA N-glycosidase activity of ricin cripples 1500 ribosomes/min and a single molecule of ricin in the cytosol can cause cell death.[11,12] Thus, ricin fusion toxins may be used in combination with bacterial fusion toxins or when bacterial fusion toxin resistance is encountered. (2) Furthermore, there is no immunologic cross-reactivity between ricin and the bacterial toxins. Patients who have been immunized with diphtheria toxin or had previous exposure to PE do not show amnestic immune responses to ricin.[13] (3) Finally, there is extensive clinical experience with RTA and blocked ricin immunotoxins suggesting safety in patients.[14] However, construction of ricin fusion toxins has been hampered by the requirement for a reducible disulfide between RTA and the ligand for cell intoxification.[15] Initial efforts to produce IL2–RTA fusions yielded nontoxic molecules. Subsequent efforts to introduce a diphtheria toxin loop peptide or factor Xa recognition sequence between IL2 and RTA did not yield disulfide-linked molecules and were noncytotoxic to IL2R bearing cells.[16]

We chose an alternative strategy for producing biologically active ricin fusion molecules. We fused oligohistidine tag or IL2 to RTB and reassociated the fusion with RTA.[17,18] Both ligand specificity (Ni^{2+} or IL2R) and heterodimer cytotoxic potency were maintained. Because we used the holotoxin in our construction, we needed to identify and modify normal tissue binding sites on ricin. Alterations were made in amino acid residues in RTB subdomains 1α and 2γ, but persistent sugar and cell binding and cell cytotoxicity were observed.[19–21] When IL2 was fused to double lectin-site mutant RTB, the reassociated heterodimer displayed reduced but continued normal tissue binding and toxicity.[22]

We recently confirmed a third lectin site in RTB subdomain 1β and reduced its sugar binding affinity by the amino acid substitution Tyr-78 to histidine (unpublished data). The triple-site mutant had 10- to 20-fold lower galactoside avidity and showed similar reduction in cell sensitivity to heterodimer. Three groups of investigators have chemically or genetically modified lectin sites on ricin and used covalently attached ligands to study cell intoxication.[23–25] In each case, reductions in lectin function led to profound decreases in cytotoxic potency. We chose to test the role of intracellular galactose binding in ricin intoxication by fusing IL2 to triple-site mutant RTB. In this report, we describe the production, chemical characterization of the fusion molecule and its reassociated heterodimer, and the biological activity of the ricin fusion protein. The results have pertinence both in understanding the molecular mechanism of ricin cytotoxicity and in the design of ricin-based fusion toxins.

Corrrespondence: AE Frankel, Hollings Cancer Center Room 311, 86 Jonathan Lucas Street, Charleston, SC 29425, USA
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Materials and methods

Construction of plasmid

Site-specific mutagenesis was performed on single-stranded pUC119-RTB[W37S/Y248H]DNA using the Sculptor *in vitro* mutagenesis kit (Amersham, Arlington Heights, IL, USA) as previously described.[19] The aromatic ring residue Tyr-78 in the 1β subdomain was changed to histidine to reduce van der Waals interactions between the protein and galactosides. The *Bam*HI–*Eco*RI mutant RTB encoding DNA fragment was subcloned into pAcGP67A plasmid (PharMingen, San Diego, CA, USA) and used to transform INVαF' *E. coli* cells (InVitrogen, San Diego, CA, USA). Transfer vector with mutant RTB was then purified by cesium chloride density centrifugation, restricted with *Bam*HI, bound and eluted from silica matrix (Promega, Madison, WI, USA), digested with calf intestinal phosphatase (Boehringer-Mannheim, Indianapolis, IN, USA), heat inactivated and repurified on silica matrix. Thre *Bam*HI fragment encoding IL2 prepared by polymerase chain reaction of pDW27 plasmid DNA as previously described[18,22] was isolated from pUC119-IL2 by digestion of cesium chloride density gradient purified plasmid with *Bam*HI, agarose electrophoresis and binding and elution from silica matrix. The 406-bp fragment was subcloned into pAcGP67A-ADP-RTB[W37S/Y248H/Y78H]. The expression vector was maintained in INVαF' *E. coli* using 100 μg/ml ampicillin. Plasmid isolated by alkaline lysis followed by cesium chloride density gradient centrifugation was double-stranded dideoxy sequenced by the Sanger method[23] using the Sequenase kit (USB, Cleveland, OH, USA).

Expression of fusion toxin

2×10^6 Sf9 *Spodoptera frugiperda* ovarian cells maintained in TMNFH medium supplemented with 10% fetal calf serum and 50 μg/ml gentamicin sulfate were co-transfected with pAcGP67A-ADP-IL2-ADP-RTB [W37S/Y248H/Y78H] DNA (4 μg) and 0.5 μg of BaculoGold AcNPV DNA (PharMingen) following the recommendations of the supplier. At 7 days post-transfection, medium was centrifuged and the supernatant tested in a limiting dilution assay with Sf9 cells and dot blots with random primer ^{32}P-dCTP labeled RTB DNA as previously described.[26] Positive wells were identified and supernatants reassayed by limiting dilution until all wells up to 10^{-8} dilution were positive. Two rounds of selection were required. Recombinant virus in the supernatant was then amplified by infection Sf9 cells at a multiplicity of infection (MOI) of 0.1, followed by collection of day 7 supernatants. Recombinant baculovirus was then used to infect 2×10^8 Sf9 cells at an MOI of 5–10 in 150 ml EXCELL400 medium (JRH Scientific, Lexena, KS, USA) with 25 mM lactose in spinner flasks. Media supernatants containing ADP-IL2-ADP-RTB[W37S/Y248H/Y78H] were collected at day 6 post-infection. Three different preparations were made.

Protein purification

Media supernatants were adjusted to 0.01% sodium azide and maintained through all purification steps at 4°C. The supernatants were concentrated 15-fold by vacuum dialysis, centrifuged at 3000 *g* for 10 min to remove precipitate, dialyzed against 50 mM NaCl, 25 mM Tris pH 8, 1 mM EDTA, 0.01% sodium azide, and 25 mM lactose (NTEAL), ultracentrifuged at 100 000 *g* for 1 h, and bound and eluted from a P2 monoclonal antibody–acrylamide matrix as previously described.[26] P2 is an anti-RTB monoclonal antibody. The affinity matrix was prepared using Ultralink azlactone functionality bis-acrylamide following the recommendations of the manufacturer (Pierce, Rockford, IL, USA). Recombinant protein was absorbed to the column in NTEAL, washed with 0.5 M NaCl, 25 mM Tris pH 9, 1 mM EDTA, 0.1% Tween 20, 0.02% sodium azide, 25 mM lactose and eluted with 0.1 M triethylamine hydrochloride pH 11. The eluant was neutralized with 1/10 volume 1 M sodium phosphate pH 4.25 and stored at –20°C until assayed. Three preparations were made.

Characterization of recombinant protein

Total protein concentration of the affinity column eluant was measured by absorbance at 280 nm. Since the optical densities of a 1 mg/ml solution of RTB and IL2 were 1.4 and 0.7, respectively, a 1 mg/ml solution of fusion protein should have a mass average optical density of 1.16. Protein was also quantitated by BioRad (Hercules, CA, USA) protein assay as per recommendations of the supplier. Aliquots of ADP-IL2-ADP-RTB[W37S/Y248H/Y78H], plant RTB and prestained low molecular weight standards were run on a reducing 15% SDS-PAGE, stained with Coomassie Blue R-250 and scanned on an IBAS automatic image analysis system (Kontron, Germany). Immunological analysis was performed using both an ELISA and immunoblot format. Costar EIA microtiter wells were coated with 100 μl of 5 μg/ml of monoclonal antibody P2, P8, or P10 reactive with RTB or monoclonal antibody to IL2, washed with PBS plus 0.1% Tween 20, blocked with 3% BSA, rewashed and incubated with samples of ADP-IL2-ADP-RTB[W37S/- Y248H/Y78H], human IL2, or plant RTB, rewashed, reacted with 1:400 rabbit antibody to ricin or 1:500 rabbit antibody to IL2, washed again, incubated with 1:5000 alkaline phosphatase conjugated goat anti-(rabbit IgG), rewashed, developed with 1 mg/ml *p*-nitrophenylphosphate in diethanolamine buffer (pH 9.6), and read on a BioRad 450 Microplate reader at 405 nm. Aliquots of ADP-IL2-ADP-RTB[W37S/Y248H/Y78H], bacterial IL2, recombinant RTB, plant RTB, and prestained low molecular weight protein standards were run on a reducing 15% SDS-PAGE, transferred to nitrocellulose, blocked with 10% Carnation's nonfat dry milk/0.1% bovine serum albumin (BSA)/0.1% Tween 20, washed with PBS plus 0.05% Tween 20, reacted with either 1:400 rabbit antibody to ricin or 1:100 mouse monoclonal antibody to IL2 (5 μg/ml), rewashed, incubated with alkaline phosphatase-conjugated goat anti-(rabbit IgG) or anti-(mouse IgG), washed again and developed with the Vectastain alkaline phosphatase kit (Vector Labs, Burlingame, CA, USA).

Reassociation with RTA

Thirty micrograms of ADP-IL2-ADP-RTB[W37S/Y248H/Y78H] were mixed with 100 μg of plant RTA in a total volume of 0.5 ml of 0.1 M triethylamine/0.1 M sodium phosphate pH 7 shaken overnight at room temperature. The reaction mixture was then analyzed by a modified ricin ELISA previously described.[22] Reassociated mixtures were also analyzed by non-reducing SDS/PAGE followed by immunoblots with P2 and P10 anti-RTB monoclonal antibodies (10 μg/ml each), monoclonal antibody to IL2 (5 μg/ml), or monoclonal anti-

body αBR12 to RTA (10 μg/ml). Densitometric scanning with the automatic image analysis system was done to quantify the shift of immunoreactive material from 50 kDa to 80 kDa.

Lectin activity of heterodimer

Asialofetuin (1 μg/ml) was bound to Costar (Cambridge, MA, USA) EIA plate wells and an ELISA was performed as previously detailed with samples of ADP-IL2-ADP-RTB[W37S/Y248H/Y78H]-RTA and castor bean ricin.[22] Briefly, the asialofetuin-coated wells were washed with PBS plus 0.1% Tween 20, blocked with 3% BSA, rewashed and incubated with 12 different concentrations of samples in EX-CELL400, rewashed and reacted with 100 μl of biotinylated αBR12 monoclonal anti-RTA antibody, rewashed and incubated with streptavidin-alkaline phosphatase, washed again and developed with *p*-nitrophenylphosphate in 50 mM diethanolamine pH 9.6. Absorbance of wells was measured at 405 nm on a microtiter plate reader. The concentration of protein giving half-maximal binding (K_d) was calculated.

IL2 receptor binding specificity

HUT102 human T leukemia cells bearing the high affinity IL2 receptor, YT2C2 human leukemia cells bearing the intermediate affinity IL2 receptor, MT-1 human leukemia cells bearing the low affinity IL2 receptor, and CEM human leukemia cells and KB human epidermoid carcinoma cells lacking the IL2 receptor were washed with PBS and attached to polylysine-coated tissue culture dishes and centrifuged at 2000 *g* for 10 min. The IL2R content of these cells was previously determined by our laboratory.[27] The cells were then incubated live at 4°C. The cells were washed with 2 mg/ml BSA in PBS and incubated in PBS plus BSA with 1 μg/ml castor bean ricin or IL2–lectin-deficient ricin. The incubation was done at 4°C. The cells were then washed with PBS and incubated with αBR12 mouse monoclonal antibody to RTA (5 μg/ml) plus BSA for 30 min at 4°C. The cells were then washed with PBS and reacted with goat anti-(mouse Ig) conjugated to rhodamine (Jackson ImmunoResearch, West Grove, PA, USA) at 25 μg/ml for 30 min at 4°C. The cells were washed again in PBS and fixed in 3.7% formaldehyde in PBS, mounted under a No. 1 coverslip in glycerol-PBS (90:10) and examined using a Zeiss (Norcross, GA, USA) axioplan epifluorescence microscope.

Cytotoxicity to mammalian cells

Measurement of protein synthesis inhibition by ricin and ADP-IL2-ADP-RTB[W37S/Y248H/Y78H]-RTA in cultured cells was done as previously described using HUT102, CEM, YT2C2, MT-1, and KB cells.[22] ADP-IL2-ADP-wild-type RTB-RTA and ADP-IL2-ADP-RTB[W37S/Y248H] prepared as described previously were also tested.[18,22] All assays were performed in triplicate. Twelve different concentrations of toxins were used. The ID_{50} was the concentration of protein which inhibited protein synthesis by 50% compared with control wells without toxin. There was no purification step after heterodimer reassociation. The free RTA concentration at the highest concentration of heterodimer in the assay (5×10^{-7} M) was 10^{-7} M. On all five cell lines, the ID_{50} for free RTA was $2–3 \times 10^{-6}$ M.[28] Thus, in the range of heterodimer ID_{50}s (10^{-8} M–10^{-12} M), the free RTA concentration (2×10^{-9} M–2×10^{-13} M) should not produce cytotoxicity.

Blocking of cytotoxicity with IL2 or lactose

HUT102 cells (1.5×10^4) were placed in sterile Eppendorf tubes at 4°C in 100 μl leucine-poor RPMI 1640 + 10% dialyzed fetal calf serum with or without 20 μg/ml IL2 or 60 mM α-lactose. Dilutions of IL2–lectin site-modified ricin and ricin at varying concentrations were added in identical medium with or without IL2 or lactose and incubated at 4°C for 30 min. Cells were pelleted at 2000 *g* for 5 min, washed once with leucine-poor RPMI 1640 + 10% dialyzed fetal calf serum, resuspended in 150 μl of the same medium and incubated at 37°C in 5% CO_2 for 24 h. ^{3}H-leucine was added as above and, 4 h later, cells were harvested with a Skatron cell harvestor and incorporated ^{3}H-leucine measured in a liquid scintillation counter. Blocking of selective cytotoxicity was estimated by comparing the ID_{50} of toxins in the presence or absence of IL2 or lactose.

Results

Yield and purity of ADP-IL2-ADP-RTB[W37S/Y248H/Y78H]

Three individual preparations from 100 ml cell supernatants were partially purified. Peak eluant fractions contained 186 μg, 228 μg and 167 μg protein based on absorbance at 280 nm. BioRad protein assay gave values of 120 μg, 160 μg, and 140 μg, respectively, for the three preparations, using bovine serum albumin standard. Densitometry of Coomassie-stained gels showed only a single detectable band at 50 kDa in each preparation (Figure 1). However, P2 antibody ELISA showed the concentration of anti-RTB immunoreactive protein was 102 μg, 49 μg, and 75 μg, respectively. Thus, purity was between 21 and 85% based on absorbance, BioRad protein assay and densitometry of Coomassie-stained gels.

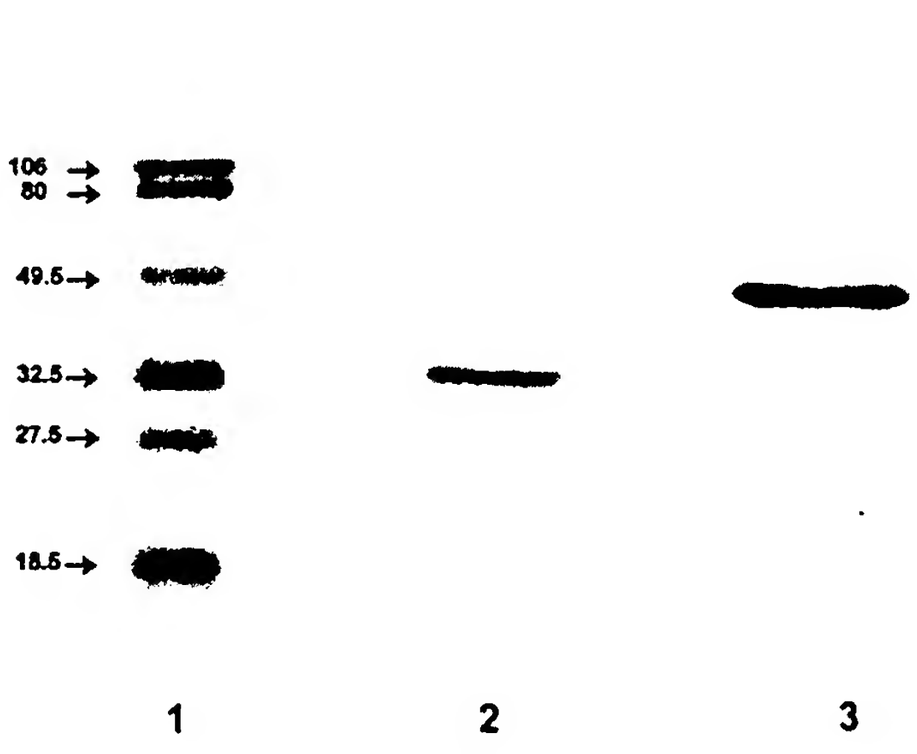

Figure 1 Coomassie-stained 15% reducing SDS-PAGE. Lane 1, low molecular weight prestained BioRad protein standards (106, 80, 49.5, 32.5, 27.5 and 18.5 kDa); lane 2, plant RTB; lane 3, ADP-IL2-ADP-RTB[W37S/Y248H/Y78H].

Immunologic cross-reactivity of ADP-IL2-ADP-RTB[W37S/Y248H/Y78H]

Different monoclonal antibodies to RTB (P2, P8 and P10) and an antibody to IL2 reacted similarly with the fusion molecule based on antibody capture ELISA. The concentration of the fusion molecule was based on a comparison of P2 antibody binding with plant RTB, so that its relative binding is taken as 100%. Antibody P8 bound 100% as well with the fusion molecule as with plant RTB. Antibody P10 bound 500% as well with IL2–lectin-deficient RTB as with plant RTB. Antibody to IL2 bound the hybrid molecule 20% as well as with recombinant human IL2 on a molar basis when an anti-IL2–anti-IL2 sandwich assay format was used. Since steric hindrance may prevent detection of bound IL2–RTB in such an ELISA, we repeated the assay using either anti-RTB with anti-IL2 detecting reagent or the reverse. In both cases, the assay then showed 100% binding relative to the ELISA using P2 capture with rabbit anti-RTB detection.

Immunoblots demonstrated reactivity with the same 52 kDa band using anti-RTB or anti-IL2 antibodies (Figure 2a and b). No weaker bands at lower molecular weight were observed with either set of antibodies suggesting the partial proteolysis found with IL2–'wild-type' RTB was not present with the lectin-deficient chimeras.

Reassociation with RTA

Two preparations of fusion toxin heterodimer were made. Under the reaction conditions (10^{-6} M of IL2–lectin-deficient RGB and 3×10^{-6} M of plant RTA, 0.1 M triethylamine/0.1 M sodium phosphate pH 7, room temperature, room air), 83% reassociation was observed in one reaction and 78% reassociation was seen in the other. The results from the sandwich ricin ELISA were similar to results from immunoblots with antibodies to RTB, RTA and IL2 (Figure 3a, b and c). Figure 3a shows the 64 kDa M_r ricin in lane 2, and both the 85 kDa M_r heterodimer and unreassociated 52 kDa M_r IL2-RTB in lane 3. Approximately, equal bands were observed. Figure 3b again shows the 64 kDa ricin in lane 2, and 85 kDa heterodimer and free 32 kDa RTA in lane 3. There was a six-fold excess of RTA relative to reassociated heterodimer as expected based on the reaction mixture. Figure 3c shows the 15 kDa recombinant IL2 in lane 3, and both 85 kDa heterodimer and 52 kDa IL2–RTB in similar amounts in lane 4. Interestingly, weaker higher molecular weight bands above the heterodimer which may represent fusion protein homodimers or aggregates were seen in both Figure 3a and c.

Lectin activity and IL2R binding of the heterodimer

ADP-IL2-ADP-RTB[W37S/Y248H/Y78H]-RTA bound immobilized asialofetuin 0.3% as well as plant ricin. The ricin K_d was 4×10^{-9} M, and the IL2 fusion toxin K_d was 1.2×10^{-7} M. Specificity for high affinity IL2 receptor was demonstrated on a live cell immunofluorescence assay (Figure 4). The IL2 fusion toxin bound to HUT102, YT2C2, MT-1 but did not bind CEM or KB cells. Binding to HUT102 cells was inhibited by IL2 but not asialofetuin. Fusion toxin binding to YT2C2 and MT-1 were 10-fold less than HUT102 cell binding consistent with the presence of lower densities of only intermediate and low affinity receptor subunits on the former cell lines.

Cell cytotoxicity

Cytotoxicities of fusion heterodimer and plant ricin for different cell lines are shown in Table 1. Ricin was uniformly toxic to all five cell lines tested with IC_{50}s of $2–3.5 \times 10^{-12}$ M. IL2–wild-type RTB–RTA was also toxic to all cell lines in the absence of lactose with IC_{50}s of $2–4 \times 10^{-12}$ M. IL2–RTB[W37S/Y248H]-RTA showed moderate specificity with IC_{50} of 4×10^{-12} M on HUT102 cells, 1.8×10^{-10} M on CEM cells and 2×10^{-10} M on KB cells. In contrast, the IL2–triple-site RTB mutant-RTA had improved specificity with an IC_{50} of 5×10^{-12} M on HUT102 cells, 1×10^{-9} M on CEM cells and 6×10^{-10} M on KB cells (Figure 5). The *in vitro* therapeutic window (the ratio of the IC_{50} of receptor negative cells to the IC_{50} of receptor positive cells) was 1 for IL2–wild-type RTB–RTA, 50 for IL2–RTB[W37S/Y248H]-RTA and 120–200 for IL2–RTB[W37S/Y248H/Y78H]–RTA.

IL2 receptor-mediated cell toxicity was tested by blocking experiments with excess IL2 or lactose (Figure 6). Excess IL2 reduced IL2–triple-site RTB mutant-RTA toxicity towards HUT102 cells by 1000-fold (IC_{50} was 1×10^{-8} M with IL2 and 1×10^{-11} M without IL2). In contrast, excess lactose had minimal effect on IL2–lectin-deficient ricin cytotoxicity (IC_{50} was 1.6×10^{-11} M with lactose and 1.4×10^{-11} M without lactose).

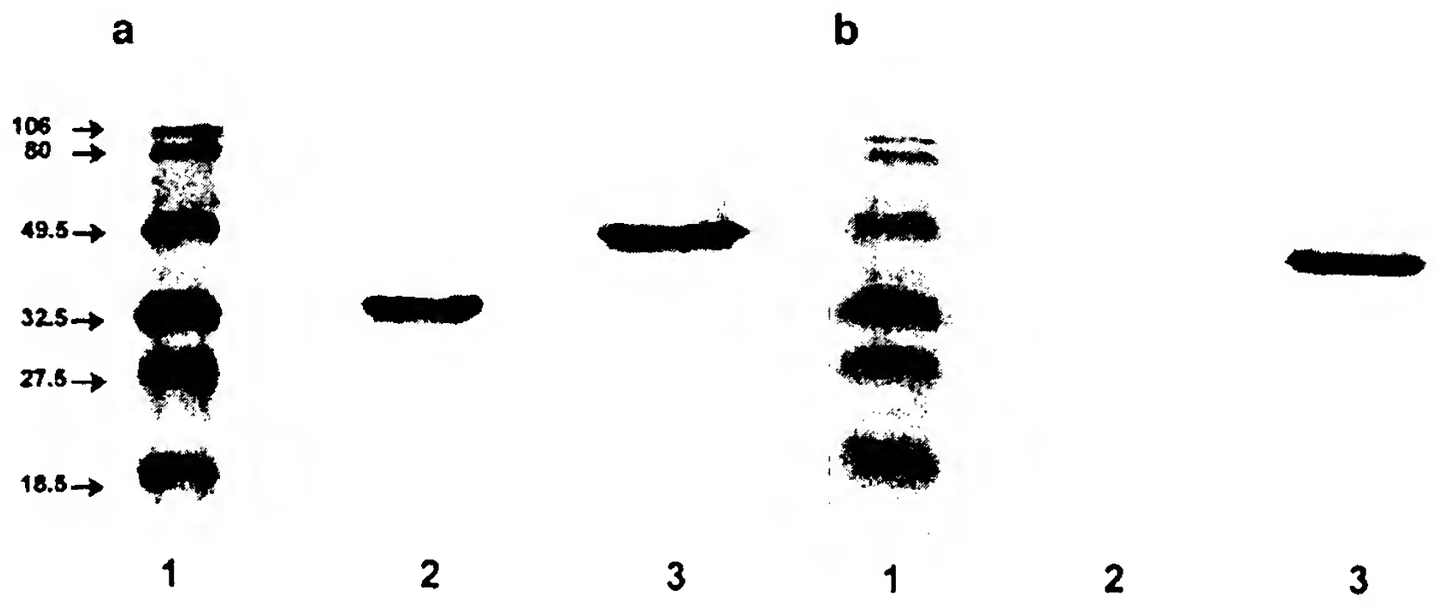

Figure 2 Immunoblot of 15% reducing SDS-PAGE. (a) Reacted with rabbit anit-RTB antibody. (b) Reacted with mouse monoclonal antibody anti-IL2. Lane 1, low molecular weight prestained BioRad protein standards; lane 2, plant RTB; lane 3, ADP-IL2-ADP-RTB[W37S/Y248H/Y78H]. Lanes identical in (a) and (b).

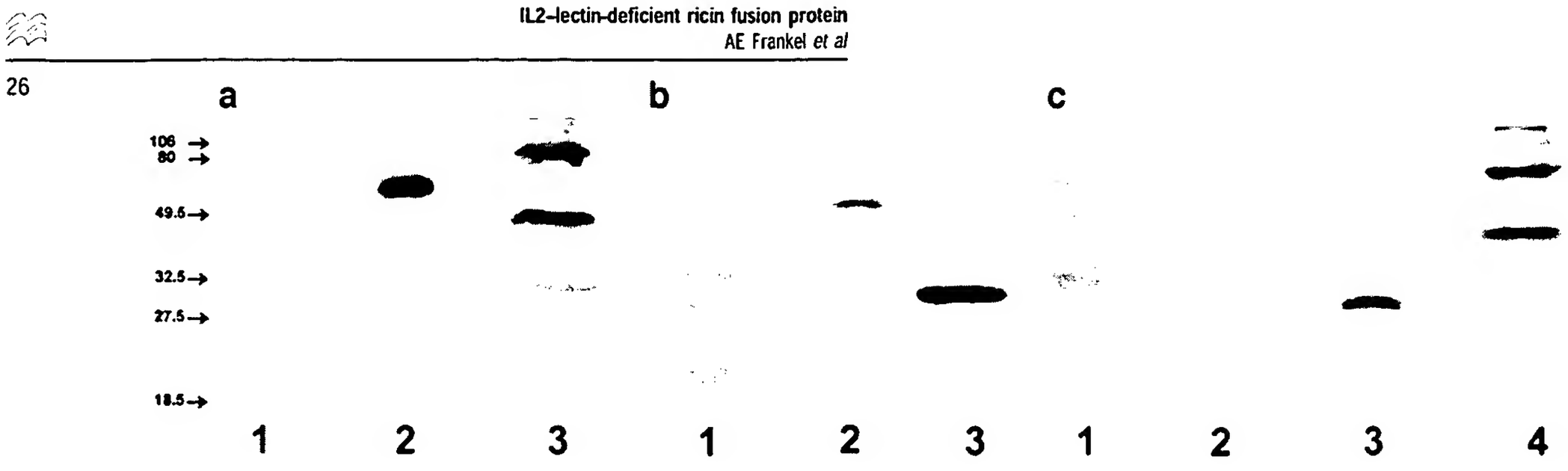

Figure 3 Immunoblots of 15% nonreducing SDS-PAGE. (a) Reacted with mouse anti-RTB monoclonal antibodies P2 and P10. (b) Reacted with mouse αBR12 anti-RTA monoclonal antibody. (c) Reacted with mouse anti-IL2 monoclonal antibody. Lanes in (a) and (b) identical. Lane, 2, low molecular weight prestained BioRad protein standards; lane 2, plant ricin; lane 3, ADP-IL2-ADP-RTB[W37S/Y248H/Y78H]-RTA. In (c), lane 1, low molecular weight prestained BioRad protein standards; lane 2, plant ricin; lane 3, recombinant human IL2; lane 4, ADP-IL2-ADP-RTB[W37S/- Y248H/Y78H]-RTA.

Discussion

Several strategies have been used to target the IL2R in patients with leukemias and lymphomas. Infusions of murine anti-IL2Rα in 7/19 adult T cell leukemia (ATL) patients produced remissions lasting from 9 weeks to 3 years.[29] To enhance clinical efficacy, both radioisotopes and peptide toxins have been linked to anti-IL2R antibodies. Yttrium-90 was conjugated to anti-IL2Rα and administered to 18 ATL patients yielding two complete and seven partial remissions with myelotoxicity.[30] PE conjugated to anti-IL2Rα produced hepatotoxicity in no responses in four ATL patients.[31] To reduce side-effects of PE conjugates and improve targeting, single chain antibodies to IL2Rα and IL2Rβ have been fused to non-cell binding PE fragments and tested for cytotoxicity on fresh leukemic blasts.[7–10] Potent selective toxicity was seen *in vitro*. RTA was conjugated to anti-IL2Rα and given intravenously to 14 Hodgkin's disease patients, and one partial remission was seen.[14,32] Vascular leak syndrome (VLS) with edema, weight gain, hypoalbuminemia and dyspnea was dose-limiting. In a parallel approach, IL2 was fused to fragments of DT[3,4] and given systemically to lymphoid malignancy patients. DAB_{486} IL2 – consisting of the first 486 amino acids of DT fused to IL2 – produced three complete remissions and eight partial remissions among 109 patients.[5] DAB_{389} IL2 – the first 389 amino acid residues of DT fused to IL2 – yielded five complete remissions and seven partial remissions among 35 cutaneous T cell lymphoma patients.[6] Mild hepatotoxicity and VLS were observed in the first cycle of treatment in some patients. Thus, results with both radioimmunoconjugates and fusion toxins showed responses in about half of patients with receptor-positive lymphoid neoplasms. However, durable complete remissions were rare suggesting the need for more potent and selective IL2R-directed therapies. Ricin protein targeted to the IL2R may provide such a more potent and selective reagent.

Synthesis of an IL2R-targeted ricin fusion protein for preclinical and clinical development requires adequate yields, simple purification, adequate stability at room temperature and 37°C, and selective toxicity to IL2R bearing lymphocytes. The IL2–triple-site mutant-RTB fusion protein was obtained at 50% purity in good yields of 0.75 mg/l culture. This compares with 1 mg/l for IL2–wild-type RTB and 0.34 mg/l IL2–double-site mutant RTB fusion protein. The IL2 protein was obtained at 50% purity in good yields of 0.75 mg/l culture. This compares with 1 mg/l for IL2–wild-type RTB and 0.34 mg/l IL2 double-site mutant RTB fusion protein. The IL2–triple-site mutant RTB molecules reacted with antibodies to IL2 and RTB both by ELISA and Western blots suggestive of maintenance of at least some IL2 and RTB epitopes on the fusion molecule. Furthermore, the protein was secreted into the insect cell medium, and purification was accomplished by a one-step immunoaffinity absorption. This contrasts with the requirement for denaturation and refolding for many bacterial toxin fusion proteins[36,37] and chemical derivatization and conjugation for immunotoxins.[38]

The toxophore domain of ricin (RTA) was added by simply mixing with the IL2 triple-site mutant RTB at 10^{-6} M. Extensive ionic and hydrophobic bonds in the RTA–RTB interface promote reassociation and disulfide bond formation.[33] Our observation of 80% reassociation compares favorably with the 60% reassociation for IL2–wild-type RTB–RTA,[18] 55% reassociation for IL2–double-site mutant RTB–RTA[22] and 50% reassociation for plant RTB–RTA under identical conditions.[26] The heterodimers were stable at high dilution (10^{-12} M) suggesting formation of the disulfide bond between RTA Cys-259 and RTB Cys-4.[34]

Binding specificity of the lectin-deficient heterodimer was demonstrated in both ELISA and cell immunofluorescence formats. The fusion toxin displayed 0.3% binding to immobilized asialofetuin. The K_d was 1.2×10^{-7} M *vs* 4×10^{-9} M for plant ricin. This weak binding compares to 1% binding ($K_d = 4 \times 10^{-7}$ M) for IL2–RTB[W37S/Y248H]–RTA and 59% binding ($K_d = 7 \times 10^{-9}$ M) for IL2–wild-type RTB–RTA. The low level binding observed in the ELISA is near the limits of detection in this assay (lower limit 0.1% relative to wild-type ricin or $K_d = 4 \times 10^{-6}$ M). Nevertheless, we believe the small residual binding of the 1α, 1β, 2γ triple mutant fused to IL2 is real and due to incomplete inactivation of one or more sites. Subdomain 1α mutation W37S reduced sugar binding avidity four-fold, while other 1α subdomain mutations (K40M and K40M/N46G) yielded proteins with seven- to eight-fold reductions in asialofetuin avidity.[19] The W37S mutation was used in the IL2–triple-site mutant because of its much better yields.

IL2 triple-site RTB mutant-RTA bound to cells possessing low, intermediate and high affinity IL2R. The lack of detectable binding to other human cell lines which still have cell surface galactosides may be due to the insensitivity of the immunofluorescence assay. A two and one-half log reduction in sugar binding may be beneath the immunofluorescence detection limit. Binding to cell lines was blocked by IL2 but not asialofetuin.

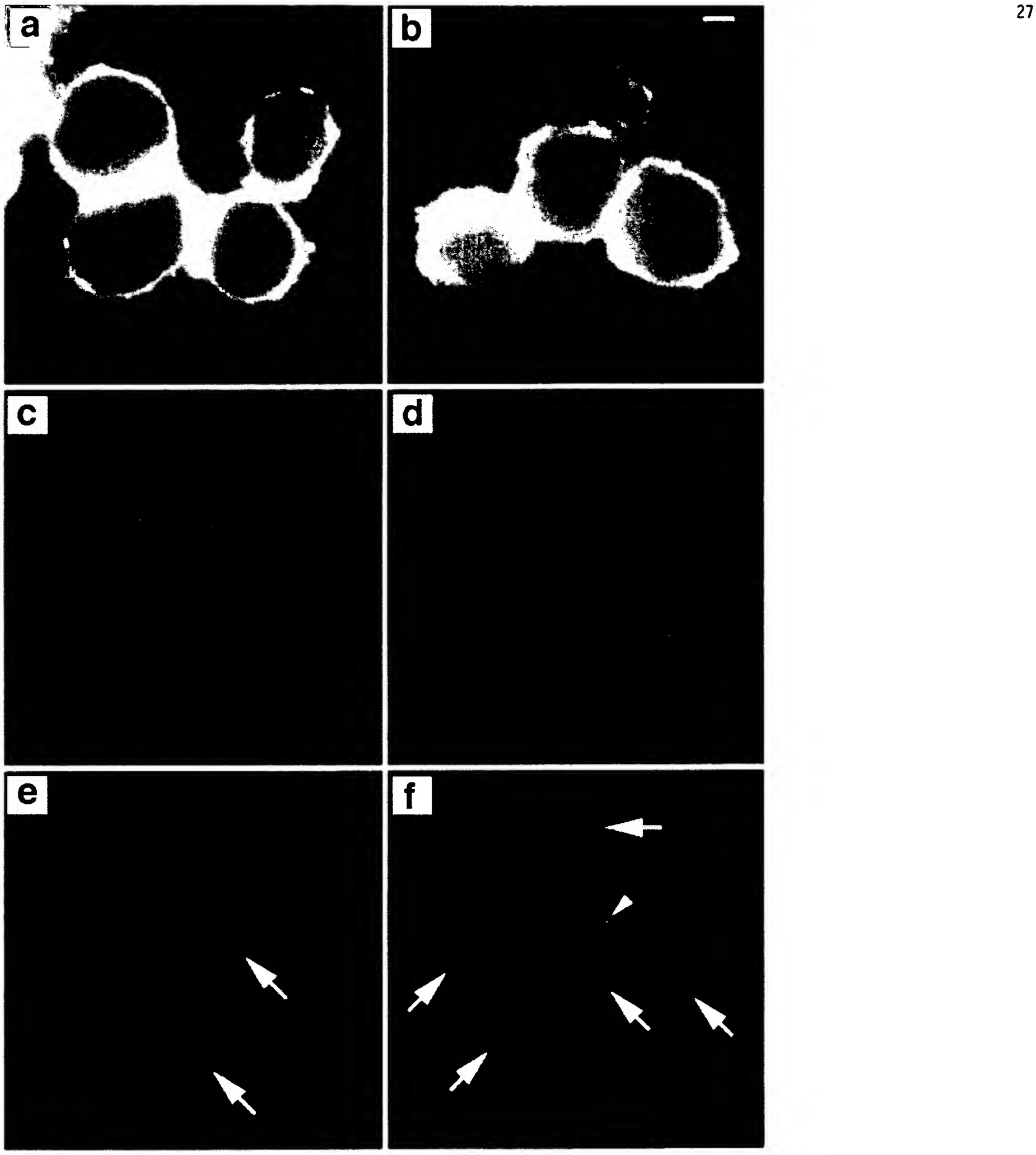

Figure 4 Binding of IL2–lectin-deficient ricin and ricin to mammalian cells. Cells were attached to poly-lysine-coated tissue culture dishes and all incubations were done at 4°C. The cells were washed with 2 mg/ml BSA in PBS and the PBS plus BSA plus 1 μg/ml of plant ricin or insect ADP-IL2-ADP-RTB[W37S/Y248H/Y78]-RTA, rewashed, incubated with 1:100 rabbit anti-ricin in PBS plus BSA, rewashed, incubated with affinity-purified goat anti-(rabbit Ig) coupled to rhodamine at 25 μg/ml, washed again and fixed in 3.7% formaldehyde in PBS. (Magnification $\times$962; bar = 6.8 μm. Positive staining is represented by uniform surface labeling.) (a) YT2C2 cells incubated with ricin 1 μg/ml as control. (b–f) with 1 μg/ml fusion toxin. (b) HUT102 cells; (c) MT1 cells; (d) YT2C2 cells; (e) KB cells; (f) CEM cells.

The most important property of an IL2–lectin-deficient ricin fusion protein is its selective cytotoxicity to IL2R expressing cells. Three previous studies suggested that, in some cases, targeted ricin molecules in which the galactose-binding sites were removed genetically or chemically lose critical intracellular intoxication functions and cannot kill cells.[23–25] Goldmacher *et al*[23] used antibody conjugates to ricin with one, two or three affinity cross-linkers. The triply cross-linked molecules lacked sugar-binding and were unable to intoxicate antigen-bearing cells. Newton *et al*[24] reassociated *Xenopus laevis* oocyte-derived RTB with modifications of two lectin sites (K40M/N46G/N255G) with plant RTA. The lectin-deficient heterodimer was nontoxic to mouse macrophages in the presence of lactose, even though binding and internalization was mediated by binding to mannose receptors. Finally, Youle *et al*[25] attached mannose-6-phosphate to tyrosyl acetylated ricin and measured cytotoxicity to mannose-6-phosphate receptor expressing fibroblasts in the presence of lactose. The

Table 1 Cell cytotoxicity of ricin fusion proteins

Cell line	*IC_{50}(M)*			
	Ricin	*IL2-WT ricin*	*IL2-DS ricin*	*IL2-TS ricin*
HUT102	2×10^{-12}	2×10^{-12}	4×10^{-12}	5×10^{-12}
CEM	4×10^{-12}	4×10^{-12}	2×10^{-10}	1×10^{-9}
KB	2×10^{-12}	2×10^{-12}	2×10^{-10}	6×10^{-10}
MT-1	2×10^{-12}	3×10^{-12}	1×10^{-11}	8×10^{-11}
YT2C2	2×10^{-12}	4×10^{-12}	9×10^{-12}	1×10^{-11}

[a]Cell cytotoxicity assays performed in triplicate as described in text. IC_{50} is the concentration of toxin reducing protein synthesis by 50% after 24 h incubation.
IL2-WT ricin, IL2-wild-type RTB-RTA; IL2-DS ricin, IL2-RTB[W37S/Y248H]-RTA; IL2-TS ricin, IL2-RTB[W37S/Y248H/-Y78H]-RTA.

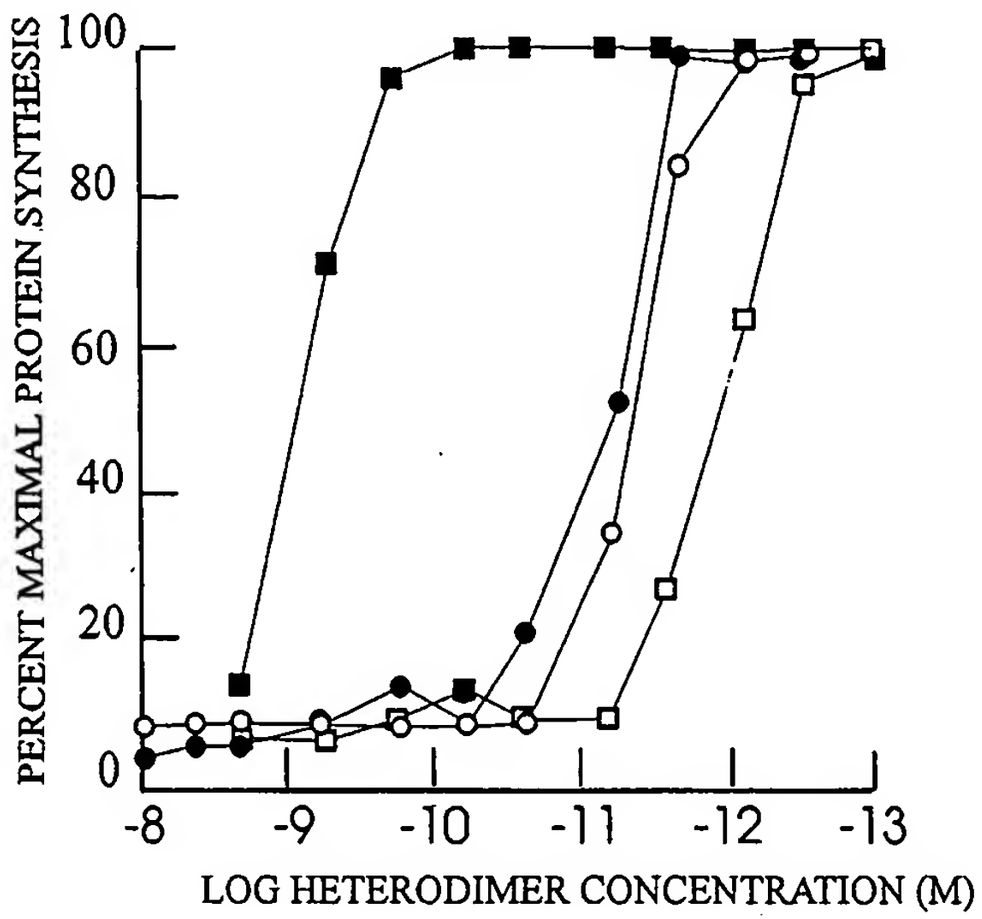

Figure 5 HUT102 cell cytotoxicity of IL2-lectin deficient ricin and ricin proteins. Cells were exposed at the dilutions indicated for 24 h at 37°C/5% CO_2. Incorporation of ^{3}H-leucine was assayed after a 4 h incubation and compared against untreated cell incorporation. (□) plant ricin without added lactose; (■) plant ricin with 100 mM lactose; (○) ADP-IL2-ADP-RTB[W37S/Y248H]-RTA without added lactose; (●) ADP-IL2-ADP-RTB[W37S/Y248H]-RTA with 100 mM lactose.

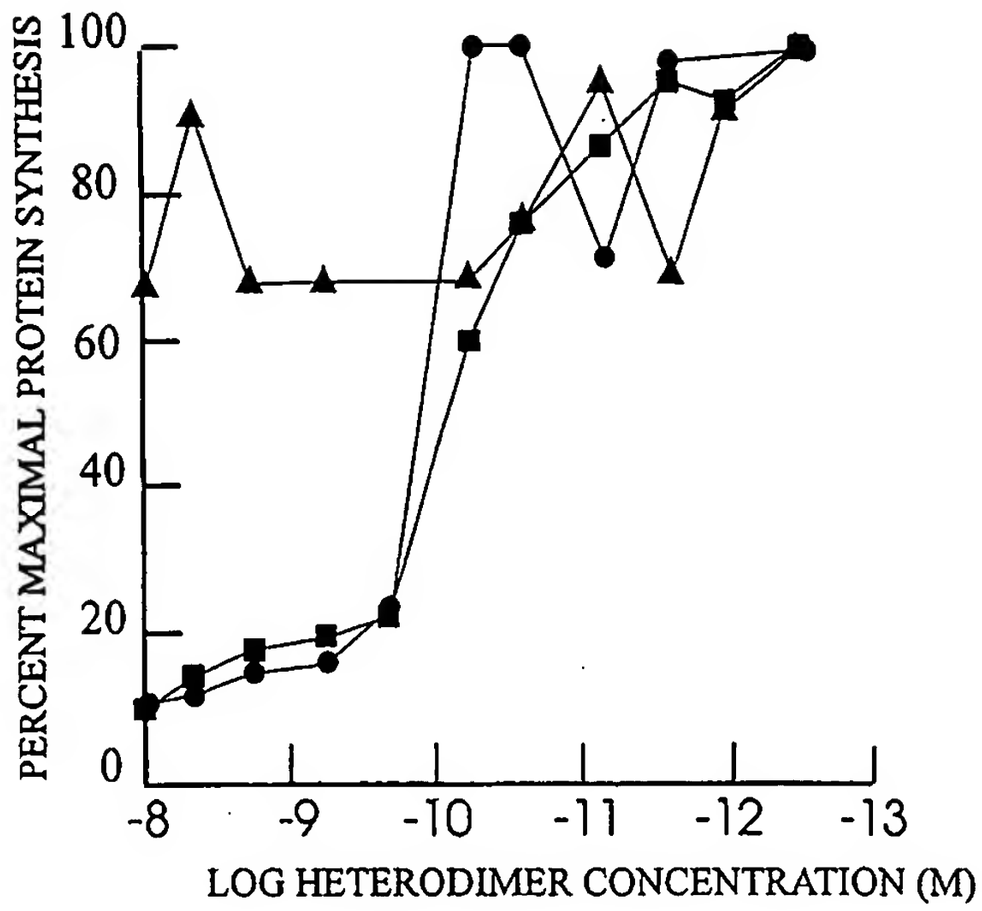

Figure 6 Blocking experiments of IL2-lectin-deficient ricin cytotoxicity to HUT102 cells by IL2 and α-lactose. In each case: (■) media alone; (●) 100 mM α-lactose added; (▲) 20 μg/ml human IL2 added. Cells were exposed at the dilutions indicated for 30 min at 4°C, washed and incubated in media alone for 24 h at 37°C/5% CO_2. Incorporation of ^{3}H-leucine was assayed after a 4 h incubation and compared against untreated cell incorporation.

lectin-deficient ricin conjugate again had reduced cell cytotoxicity, although cell binding and entry was mediated by non-galactoside mechanisms. If IL2R-targeted ricin behaved similarly, we would expect markedly reduced cytotoxicity for the IL2-triple-site RTB mutant-RTA. However, Kronke *et al*[39] and Winkler *et al*[40] demonstrated RTA alone conjugated to monoclonal anti-IL2Rα subunit antibodies yielded conjugates toxic to ATL and Hodgkin's disease cells, respectively, with IC_{50}s of 2×10^{-10} M and 6×10^{-12} M. These previous findings suggested the fusion toxin would kill IL2R bearing cells, albeit with variable potency. The lectin-deficient fusion toxin was equally toxic as IL2–wild-type RTB–RTA or ricin. Thus, either intracellular galactose binding is not required for cell intoxication by ricin directed into cells by the IL2R, or the minimal residual galactose binding of the triple-site mutant has adequate avidity for the intracellular galactose-binding step. We intend to test the former hypothesis by evaluating the cytotoxicity of RTB[W37S/Y248H/Y78H]–RTA on mouse macrophages. The latter hypothesis requires further reduction in ricin binding which we will study by preparing additional triple-site and quadruple-site RTB mutants. Fortunately, the potent cytotoxicity of the IL2–triple-site RTB mutant-RTA is beneficial for preclinical development, and rodent toxicity studies are ongoing.

The IL2–lectin-deficient fusion toxin was selectively cytotoxic to hematopoietic neoplastic cell lines with the heterotrimeric high affinity IL2R. The molecules were less toxic to cells with intermediate or low affinity IL2R, and over 100-fold less toxic to cells without IL2R. This therapeutic window is not as wide as the 1000-fold difference in cell sensitivity observed for ricin cross-linked with dichlorotriazine derivatized triantennary glycopeptide ligands.[41] Upon conjugation of the chemically blocked ricin to anti-CD19 antibody initially a 1000-fold difference in sensitivity between receptor positive and negative cells was observed.[42] However, subsequent preparations of purified doubly blocked ricin anti-CD19 immunoconjugate yielded sensitivity of receptor negative cells to anti-CD19-blocked ricin only 100-fold less than receptor positive cells (5 nM *vs* 0.05 nM).[43] The difference in therapeutic window was ascribed to variations in the content of doubly blocked and triply blocked immunoconjugate in the preparations. In one study with anti-CD25-RTA, cells lacking the IL2R were also 100-fold less sensitive to drug.[39] The *in vitro* therapeutic index was not determined for another anti-CD25-RTA.[40] The sensitivity of cells bearing a portion of the IL2R were similar to those seen with IL2-PE40[35] but distinct from those seen with DAB_{389} IL2.[27] The latter molecule fails to intoxicate low affinity IL2Rα,γ cells perhaps due to steric effects of the N-terminal toxin moiety. As a note of caution, fresh leukemic blasts often display lower levels of IL2Rα and IL2Rβ than cell lines and may show lowered sensitivity to the ricin fusion molecule.[8]

Competition experiments with excess ligand demonstrated that IL2, but not lactose, inhibited cell cytotoxicity of the IL2–triple-site RTB mutant-RTA. Thus, the fusion toxin needs IL2R binding for cell intoxication.

Why prepare an IL2–ricin fusion toxin, since highly active monoclonal antibody ricin A chain and blocked ricin conjugates are available? Although preclinical *in vitro* and *in vivo* animal model results with antibody ricin conjugates have been excellent,[32] clinical studies have shown at best 20–40% transient partial remission both in non-Hodgkin's lymphoma and Hodgkin's disease.[13,14] The modest efficacy was also associated with serious vascular leak syndrome. Prolonged exposure of vascular endothelium to toxin conjugates may lead to injury. Poor capillary permeability secondary to large conjugate molecular weight (200 kDa) may exacerbate vascular damage and limit exposure of extravascular leukemic cells to drug. Furthermore, immune responses to both mouse antibody and toxin may limit treatments in at least some patients. The fusion toxin technology may partially circumvent some of these clinical pharmacologic barriers. The drug is smaller and the cytokine moiety should be nonimmunogenic permitting better access of drug to leukemic blasts. The homogeneity of the reagent reduces variability in drug size and activity. Finally, while the interleukin 2 receptor system may not require holotoxin enhancement functions, the IL2–ricin fusion molecule may serve as a model for other cytokine and single chain antibody ricin fusion therapeutics for leukemia which may possess less potent RTA immunoconjugates.

This report describes a novel IL2R-targeted ricin fusion protein worthy of testing in animal models of leukemia, lymphoma and autoimmune diseases with comparisons to other IL2R-directed therapeutics. The molecule also provides an engineering model for construction of other plant toxin fusions with other hormones and single chain antibodies for therapy of a number of malignancies and autoimmune disorders.

Acknowledgements

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Antib dy-targeted interleukin 2 stimulates T-cell killing of autologous tumor cells

(recombinant antibody/cytokine fusion protein/tumor-infiltrating lymphocytes)

STEPHEN D. GILLIES*†, EDWARD B. REILLY*, KIN-MING LO*, AND RALPH A. REISFELD‡

*Research Department, Abbott Biotech, Inc., 119 Fourth Avenue, Needham Heights, MA 02194; and ‡Department of Immunology, The Scripps Research Institute, 10666 North Torrey Pines Road, La Jolla, CA 92037

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ABSTRACT A genetically engineered fusion protein consisting of a chimeric anti-ganglioside GD2 antibody (ch14.18) and interleukin 2 (IL2) was tested for its ability to enhance the killing of autologous GD2-expressing melanoma target cells by a tumor-infiltrating lymphocyte line (660 TIL). The fusion of IL2 to the carboxyl terminus of the immunoglobulin heavy chain did not reduce IL2 activity as measured in a standard proliferation assay using either mouse or human T-cell lines. Antigen-binding activity was greater than that of the native chimeric antibody. The ability of resting 660 TIL cells to kill their autologous GD2-positive target cells was enhanced if the target cells were first coated with the fusion protein. This stimulation of killing was greater than that of uncoated cells in the presence of equivalent or higher concentrations of free IL2. Such antibody–cytokine fusion proteins may prove useful in targeting the biological effect of IL2 and other cytokines to tumor cells and in this way stimulate their immune destruction.

Much attention has been focused on the use of interleukin 2 (IL2) for cancer immunotherapy because of its stimulatory effect on a broad range of immune cell types, including both T and B cells, monocytes, macrophages, and natural killer cells. One class of cells resulting from *in vitro* or *in vivo* stimulation of immune cells has been called lymphokine-activated killer cells (1), and therapeutic approaches using such populations have shown clinical responses with some tumor types (2). Other, more refractory tumors may show greater responses if monoclonal antibodies directed against these tumors are used in combination with IL2 (3, 4). Such antibodies mediate antibody-dependent cellular cytotoxicity (ADCC) through their interactions with both the tumor cell antigen and the Fc receptor (CD16) present on certain subsets of natural killer cells, monocytes, granulocytes, and macrophages.

While IL2 treatment *in vivo* leads to increases in both natural killer and ADCC activities, the cytolytic activity of antigen-specific, major histocompatibility complex (MHC)-restricted T cells may actually be reduced (5). Treatment of T cells with anti-CD3 antibody prior to IL2 exposure greatly increases T-cell cytolytic activity (6). Likewise, expansion of tumor-infiltrating lymphocytes (TIL) by culture in the presence of high concentrations of IL2 with periodic target-cell stimulation leads to substantial increases in cytolytic activity (7). Both approaches involve costimulation of IL2 and T-cell antigen receptors for expansion and maintenance of T-cell cytolytic activity. Thus, an optimal therapy might combine IL2 activation and tumor antigen presentation together with a tumor-specific antibody that mediates both complement-dependent cytotoxicity (CDC) and ADCC activities. By combining a chimeric anti-ganglioside GD2 antibody (ch14.18) which has potent CDC and ADCC activities (8), with IL2, we hope to target this cytokine to tumors such as neuroblastoma (9, 10) and melanoma (11) expressing GD2. In this way, relatively large amounts of tumor antigens should be present during IL2 activation for expansion of cytotoxic T cells, since melanoma cell lines have been reported to express an average of 1.5×10^7 sites per cell for ch14.18 (8). Furthermore, the antibody would also be available to target Fc receptor-bearing cells that have been activated by the targeted IL2.

The ch14.18 antibody used in this report has already been shown to mediate potent ADCC activity by IL2-activated peripheral blood mononuclear cells from cancer patients (12). We have focused on the ability of a ch14.18–IL2 fusion protein to stimulate the proliferation and cytolytic activity of a human T-cell line against autologous melanoma targets. This cell line, 660 TIL, is $CD3^+$, $CD8^+$, antigen-specific, and MHC class I-restricted and was originally obtained by outgrowth from a human metastatic melanoma (13). A melanoma line, 660 mel, was derived from the same tumor and serves as a source for antigen stimulation and as an autologous target for 660 TIL (14). Results of this study show that tumor cells coated with a fusion protein in which IL2 is at the carboxyl terminus of the heavy-chain constant region 3 (CH3) exon of ch14.18 (CH3–IL2) efficiently stimulate resting 660 TIL cells to kill autologous targets. These coated cells serve as a model for tumors that have been targeted *in vivo*.

MATERIALS AND METHODS

Plasmid Constructs. The immunoglobulin–IL2 fusion protein expression vector was constructed by fusing a synthetic human IL2 sequence to the carboxyl end of the human $C_\gamma 1$ gene. A synthetic DNA linker, extending from the *Sma* I site near the end of the antibody coding sequence to the single *Pvu*II site in the IL2 sequence, was used to join the amino-terminal codon of mature IL2 to the exact end of the CH3 exon (CH3–IL2). The fused gene was inserted into the vector pdHL2-14.18 as described earlier for an antibody–lymphotoxin fusion protein construct (15). Additional constructs were made in which the IL2 sequence was fused to the *Sac* I site in the hinge region of the human $C_\gamma 3$ gene (Fab–IL2) or to the end of the CH2 exon at a *Taq* I site (CH2–IL2). In both cases synthetic linkers were used to fuse the antibody and IL2 sequences directly without introducing any additional amino acid residues.

Transfection and Purification. The expression plasmids were introduced into Sp2/0-Ag14 cells by protoplast fusion and selected in Dulbecco's modified Eagle's medium (GIBCO) containing 10% fetal bovine serum and 100 nM

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Abbreviations: ADCC, antibody-dependent cellular cytotoxicity; CDC, complement-dependent cytotoxicity; IL2, interleukin 2; MHC, major histocompatibility complex; TIL, tumor-infiltrating lymphocyte(s); C, constant; CH, heavy-chain C region; V, variable.

†To whom reprint requests should be addressed.

methotrexate. Clones secreting the fusion proteins were identified by ELISA (16). The highest producers were grown in increasing concentrations of methotrexate and subcloned in medium containing 5 μM methotrexate. The CH3–IL2 fusion protein was purified using protein A-Sepharose (Repligen, Cambridge, MA) as affinity adsorbent. Small amounts of the Fab–IL2 and CH2–IL2 proteins were purified using an anti-human κ chain immunoaffinity column.

Antigen-Binding Activity. The antigen-binding activity was measured in a solid-phase ELISA using a chloroform extract of human neuroblastoma cells as a source of GD2 (17). In some cases the fusion proteins were first treated with plasmin (0.125 casein unit/ml) in 50 mM Tris, pH 8/150 mM NaCl for 1–2 hours at 37°C. Aprotinin (Sigma) was added at the end of the digestion (200 kallikrein inhibitory units/ml) when the digested protein was tested for antigen binding or IL2 activity.

Human TIL Culture. The 660 TIL line and its autologous $GD2^+$ tumor line 660 mel were established from a human melanoma tumor sample and maintained in culture as described (14).

IL2 Assays. IL2 activity of antibody–IL2 fusion proteins was assayed in standard T-cell proliferation assays using either the mouse CTLL-2 line (18) or 660 TIL. After IL2 depletion for 48 hr, 3×10^4 CTLL-2 cells or 10^5 660 TIL cells were added to individual wells of a 96-well microtiter plate in a volume of 0.2 ml with various concentrations of fusion protein (normalized for IL2 content) or recombinant IL2 [either yeast-derived (Genzyme) or bacteria-derived (Hoffmann–La Roche)]. After 72 hr, 0.5 μCi (18.5 kBq) of [*methyl*-^{3}H]thymidine was added to each well, and plates were harvested 12 hr later. All samples were tested in duplicate.

Cytotoxicity Assays. Cytolytic activity of 660 TIL was measured in ^{51}Cr-release assays against 660 mel target cells. The 660 TIL cells were depleted of IL2 for 4 days prior to their use in assays except where noted. Target cells (3×10^6) were labeled with 300 μCi of $Na_2{}^{51}CrO_4$ (Amersham) for 1 hr at 37°C and washed in RPMI 1640 with 10% heat-inactivated fetal bovine serum. For experiments in which target cells were coated with antibody, ^{51}Cr-labeled target cells (10^6 in 1 ml) were incubated with ch14.18 or CH3–IL2 fusion protein (50 μg/ml in RPMI 1640 with 10% heat-inactivated fetal bovine serum). After 1 hr at 4°C with periodic mixing, cells were washed three times with serum-containing medium to remove excess antibody and were used in cytotoxicity assays. In some experiments the effect of adding either antibody or IL2 at the time of assay was determined. Duplicate assay mixtures were incubated at 37°C for 7–16 hr.

RESULTS

Characterization of Immunoglobulin–IL2 Fusion Proteins. Several forms of antibody–IL2 fusion proteins were constructed and expressed in transfected hybridoma cells. In initial studies we compared antigen-binding and IL2 activities of constructs consisting of a chimeric light chain, expressed in the same transfected cell with various truncated heavy chain–IL2 fusion proteins. In one case IL2 was fused to the beginning of the first hinge domain of the human $C_\gamma 3$ gene (deleting CH2 and CH3 exons) and in another construct IL2 was fused to the end of the CH2 exon (deleting CH3; Fig. 1*A*). These heavy-chain fusion protein constructs were expressed together with the variable (V) regions of the anti-GD2 antibody 14.18 and the human C_κ gene. Secreted heavy chains were found to associate with the chimeric light chain to form Fab–IL2 or CH2–IL2 fusion proteins, but the latter did not assemble into a whole antibody even though it contained an intact hinge region (Fig. 1*B*). The covalent disulfide bonds that are normally involved in inter-heavy-chain binding are contained in the hinge.

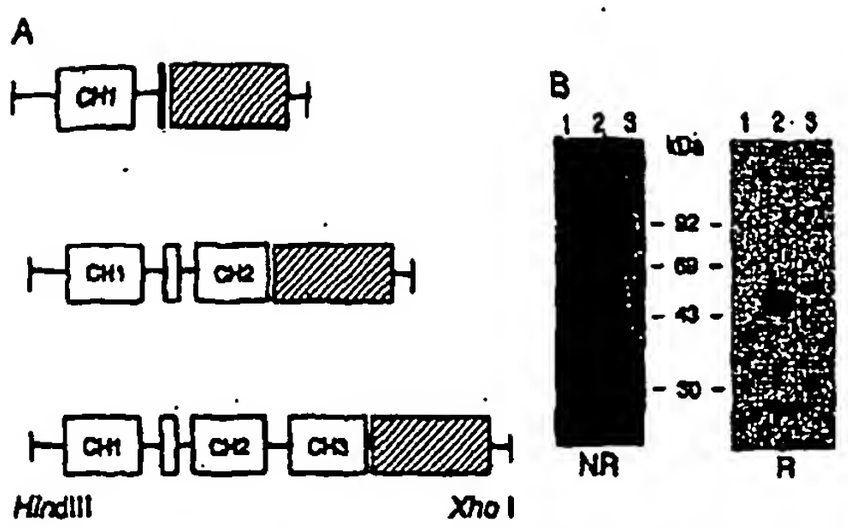

FIG. 1. Construction of fusion protein genes and analysis of the expressed proteins. (*A*) IL2 sequence (hatched box) was joined to the human $C_\gamma 3$ (Fab–IL2) or $C_\gamma 1$ heavy-chain gene by using synthetic DNA linkers, to express (from top to bottom) Fab–IL2, CH2–IL2, and CH3–IL2 constructs. Unlabeled open box represents the hinge region. (*B*) Gel electrophoretic analysis of Fab–IL2 expressed with B72.3 V regions (lanes 1); chimeric 14.18 whole antibody (lanes 2), and CH2–IL2 expressed with 14.18 V regions (lanes 3). Samples either were nonreduced (NR) or were reduced (R) with 2-mercaptoethanol. The gel was stained with Coomassie blue.

The antigen-binding and IL2 activities of these proteins are summarized in Table 1. The Fab–IL2 protein containing the 14.18 V regions had no antigen-binding activity (within the limits of our binding assay), whereas the CH2–IL2 protein was strongly positive. The 14.18 Fab, produced by genetic engineering, had greatly reduced antigen binding (data not shown), suggesting that bivalency is required for full activity. We then constructed a second Fab–IL2 fusion protein using the V regions of the anti-TAG 72 antibody B72.3 (19), to test whether such a molecule could be made that retained both IL2 activity and antigen binding. The B72.3 Fab–IL2 protein had normal antigen-binding activity, and all of the fusion proteins had IL2 specific activities ranging from 5 to 6.5×10^6 units/mg when normalized for IL2 content.

We next decided to construct a whole antibody–IL2 fusion protein by fusing the coding sequence of IL2 to the end of the heavy-chain CH3 exon (CH3–IL2). In this way we hoped to produce fully assembled IL2 fusion proteins that might also have more favorable pharmacokinetic properties *in vivo* and that could most likely be purified by protein A-Sepharose affinity chromatography. The CH3–IL2 protein constructed with the 14.18 anti-GD2 V regions was found to be expressed as a fully assembled antibody fusion protein (Fig. 2 *Inset*) with full IL2 activity (see below) and to have enhanced antigen-binding activity. Since the carboxyl-terminal lysine residue of the heavy chain was contained in this construct (just before the +1 residue of mature IL2), we tested whether this site would be accessible to cleavage with proteases such as plasmin, which cleaves after lysine or arginine. The CH3–IL2 protein was treated with plasmin and subsequently analyzed by SDS/polyacrylamide gel electrophoresis.

Table 1. IL2 activity of immunoglobin–IL2 fusion proteins

Construct	V region	Antigen binding	IL2 activity
Fab–IL2	14.18	–	5.0×10^6
Fab–IL2	B72.3	+	6.0×10^6
CH2–IL2	14.18	+	6.5×10^6

IL2 activity in culture supernatants (without methotrexate) was determined by thymidine incorporation into mouse CTLL-2 cells. The amount of each fusion protein was determined by ELISA and the activity is reported as units/mg of IL2. Antigen binding was determined using GD2-coated (14.18) or mucin-coated (B72.3) plates as described (13).

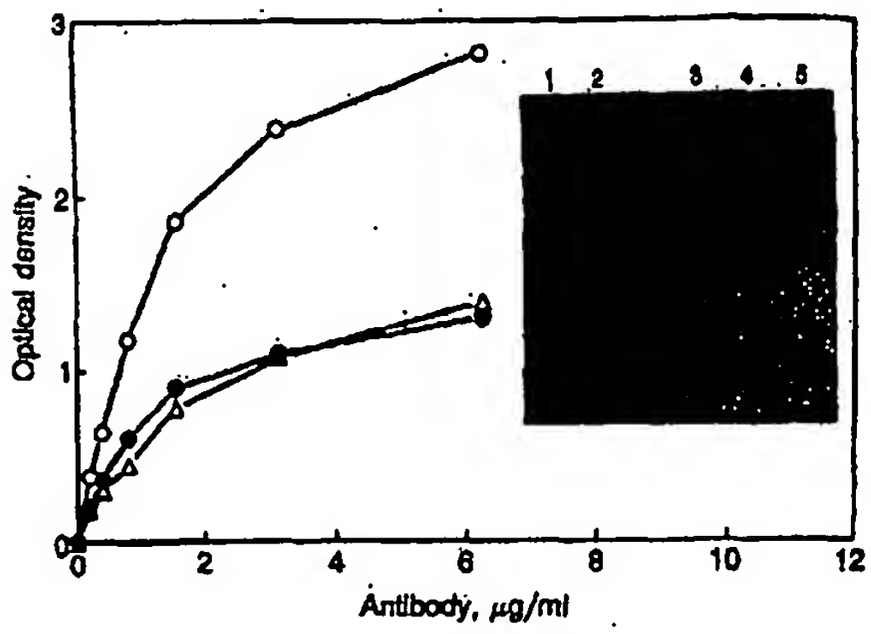

FIG. 2. Antigen-binding activity of CH3–IL2 (14.18) fusion protein before and after proteolytic cleavage to remove IL2. Purified CH3–IL2 was assayed for direct antigen-binding activity on GD2-coated plates before (○) and after (△) treatment with plasmin for 1 hr at 37°C. ; Control. (*Inset*) Electrophoretic analysis of ch14.18 (lanes 1 and 3), CH3–IL2 (lanes 2 and 4), and plasmin-treated CH3–IL2 (lane 5) under nonreducing (lanes 1 and 2) or reducing (lanes 3–5) conditions. The unlabeled lane contained molecular size markers (see Fig. 1 for sizes).

The untreated CH3–IL2 protein had much higher binding activity in a direct antigen-binding assay than the chimeric antibody, and this enhanced activity was lost when IL2 was cleaved from the heavy chain with plasmin (Fig. 2). The gel analyses showed that the antibody itself was resistant to plasmin cleavage and that a heavy chain of the expected size was generated by cleavage at the antibody/IL2 junction. These results suggest that the fused IL2 domain actively interacts in some way with the antibody to alter antigen-binding activity. Upon removal of IL2, the normal level of activity is restored.

Since the CH3–IL2 construct was fully assembled into an antibody fusion protein, it is likely that this molecule has the most favorable properties for both *in vitro* and *in vivo* studies. It could also be readily purified by affinity chromatography on protein A-Sepharose. The availability of a matched set of TIL and its autologous tumor cell line, expressing the GD2 antigen, has allowed us to exploit this system as a model for testing the biological properties of antibody-targeted IL2. Such a system was not available for a tumor cell expressing the TAG 72 antigen. For these reasons we have focused our studies on the characterization of the ch14.18 CH3–IL2 fusion protein.

Biological Activities of Whole Antibody–IL2 Fusion Proteins. The IL2 activity of the CH3–IL2 (14.18) fusion protein was tested in a standard T-cell proliferation assay using either the mouse CTLL-2 line or a human TIL line (660 TIL) established from a metastatic melanoma. Both lines were cultured without IL2 for 48 hr prior to assay. The activity of the fusion protein was found to be somewhat less than that of a recombinant IL2 made in bacteria but was identical to that of a recombinant IL2 preparation produced in yeast (2.5×10^6 units/mg) when either the murine or the human T cells were used (Fig. 3). Thus, fusion of this cytokine at the carboxyl terminus of an antibody or antibody fragment does not significantly reduce its activity. We reported a similar result when we found that lymphotoxin (tumor necrosis factor β) retained its full activity when fused to the end of the CH3 domain (15). However, in that case some inactivation of lymphotoxin activity occurred during the elution from the protein A-Sepharose column. In contrast, the CH3–IL2 preparation used in this study was stable throughout the purification and during subsequent storage for up to 18

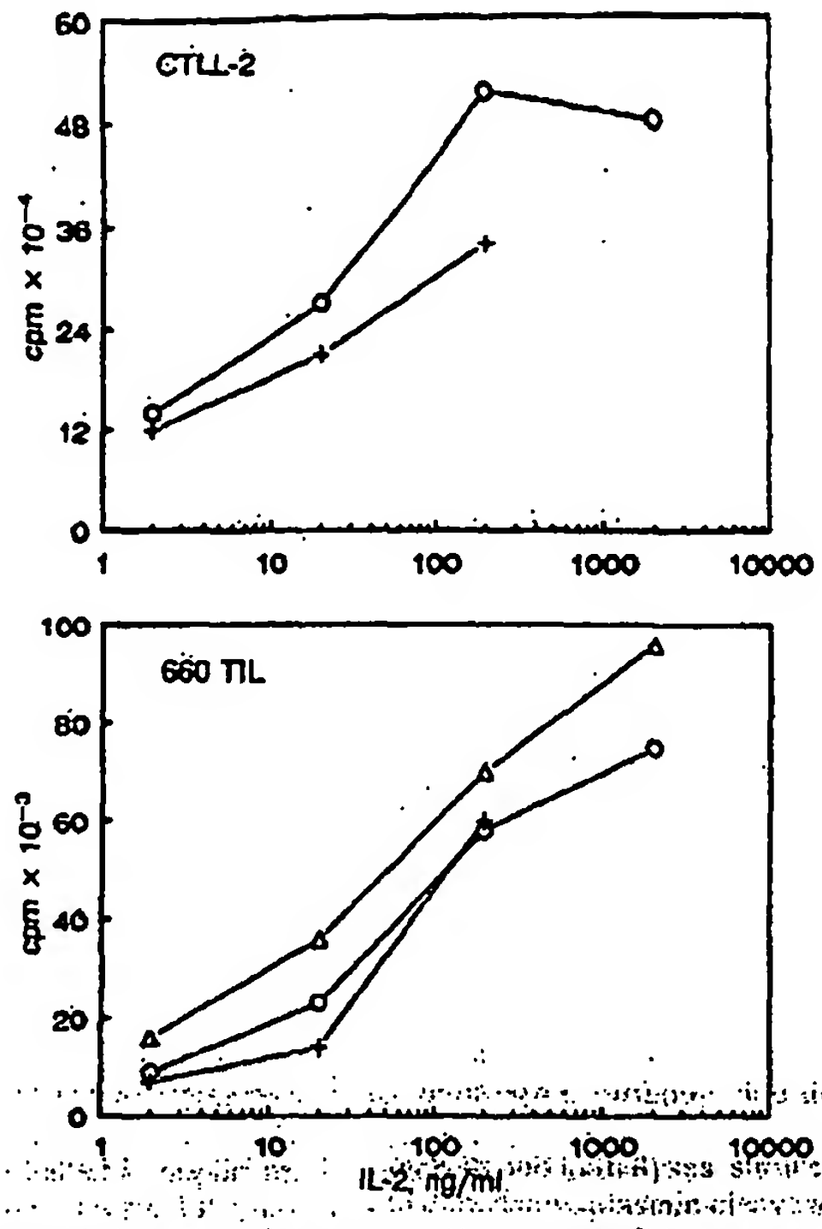

FIG. 3. IL2 activity of purified CH3–IL2 protein (○) as measured by [^{3}H]thymidine incorporation of mouse CTLL-2 (*Upper*) or human 660 TIL (*Lower*) T cells. Recombinant IL2 expressed in *Escherichia coli* (△) or in yeast (+) was used as control. Results were normalized to the IL2 content of the fusion protein.

months at −20°C. The effector functions of the CH3–IL2 protein—i.e., the ability to mediate complement and Fc receptor-dependent lysis—were also tested and found to be maintained (but somewhat decreased) when compared with that of the chimeric 14.18 antibody (data not shown). A similar result was reported for the CH3–lymphotoxin fusion protein (15).

Enhanced TIL Cytotoxic Activity of Autologous Tumor Targets. The human 660 TIL line was used to test the ability of the CH3–IL2 (14.18) fusion protein to stimulate the killing of GD2$^+$ autologous melanoma tumor cells (660 mel). The 660 TIL line is routinely cultured in serum-free medium containing IL2 (1000 units/ml) and is stimulated bimonthly with 660 mel to maintain killing activity (13). The lytic activity of this CD8$^+$ cell line for its target varies over time in culture as a function of antigen stimulation. For the purpose of this study we have also examined the effect of IL2 depletion on TIL cytotoxic activity and how this might be affected by subsequent addition of IL2 or the CH3–IL2 fusion protein. Consequently, the level of killing varies from one experiment to another, as does the ability of IL2 to enhance the killing in both normal and IL2-depleted cell cultures.

An example of a killing assay performed with 660 TIL shortly after antigen stimulation is shown in Fig. 4*A*. The tumor target cells were first coated with the fusion protein or with ch14.18 antibody and then used as targets in a 7-hr ^{51}Cr-release assay. At the higher effector/target ratio (50:1), the antibody alone stimulated killing, but to a much lesser extent than CH3–IL2. The effect of CH3–IL2 was more pronounced with TIL that had been deprived of IL2 for 4 days.

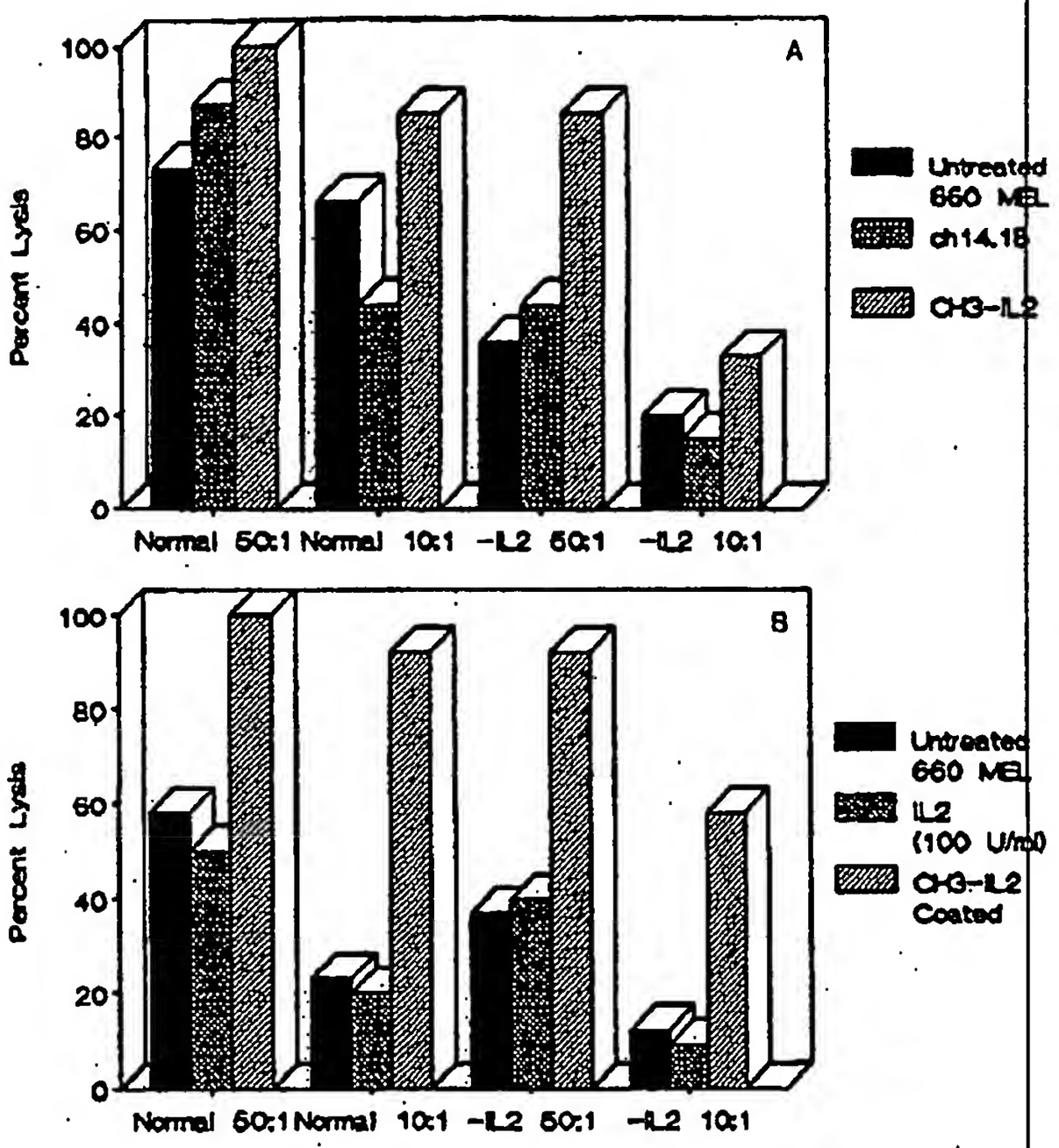

FIG. 4. Stimulation of autologous cytolytic activity by CH3–IL2-coated tumor cells. (*A*) Freshly stimulated 660 TIL cells were used as effectors against 660 mel targets in a 7-hr ^{51}Cr-release assay. Before the assay, the target cells were either untreated (filled bars) or coated with ch14.18 (cross-hatched bars) or CH3–IL2 (hatched bars). The effector cells (660 TIL) were taken from growing cultures (normal) or were cultured for 4 days without IL2 (−IL2) and were used at an effector/target ratio of 50:1 or 10:1. (*B*) Normal and IL2-depleted 660 TIL cells, 1 week after antigen stimulation, were used as effectors against untreated (filled bars) or CH3–IL2-coated (hatched bars) 660 mel targets at the indicated effector/target ratio in a 16-hr release assay. One set was incubated with added IL2 [100 units (U)/ml; cross-hatched bars].

A similar experiment comparing the fusion protein and exogenously added IL2 was performed 1 week later, when the autologous killing activity had declined. For this reason the duration of the cytotoxicity assay was extended to 16 hr. As seen in Fig. 4*B*, the addition of IL2 (100 units/ml) to the assay mixture had little effect. The stimulatory effect of CH3–IL2 in these experiments was quite striking, especially at the lower effector/target ratios and when IL2-depleted effector cells were used. The results were less pronounced once the TIL had been cultured without antigen stimulation with 660 mel tumor cells (data not shown). In all cases, the amount of stimulation obtained by coating the tumor cells exceeded that obtained by adding equivalent levels of IL2.

DISCUSSION

A fusion protein consisting of an intact tumor-specific chimeric antibody and human IL2 (CH3–IL2) has been shown to retain both antibody and IL2 functions. IL2 activity was measured by the ability of the fusion protein to stimulate the proliferation of resting human and mouse T cells. Constructs containing smaller portions of the antibody molecule were also found to retain full IL2 activity. These results contrast with an earlier report (20) in which a purified Fab–IL2 fusion protein was 200-fold less active than recombinant IL2 in a proliferation assay. Our constructs also differ from those reported by Fell *et al.* (20) in that we have directly fused antibody and IL2 sequences, without the introduction of artificial linker residues. In the case of our 14.18 Fab–IL2, we could not demonstrate antigen binding, but this was not likely due to the fusion of IL2, since a similar construct made with the V regions of B72.3 maintained both IL2 and antigen binding activities. Genetically engineered 14.18 Fab was also found to have greatly reduced antigen-binding activity.

Melanoma cells expressing GD2 that have bound the CH3–IL2 fusion protein serve as much better targets for cytotoxic T cells. This was demonstrated by using a TIL line (660 TIL) that had been maintained in culture in the presence of high concentrations of IL2 with periodic antigen stimulation by autologous melanoma cells (660 mel). The stimulatory effect was most pronounced when the TIL had been maintained without IL2 for several days. The amount of IL2 that would be bound to the 660 mel cells under saturating conditions would be equivalent to 50 units/ml in the assay, if these cells express $>10^7$ GD2 sites as was reported for M21 melanoma cells (8). These levels of IL2 added to the assay mixture were less effective than the fusion protein in stimulating cytolytic activity.

In the experiment depicted in Fig. 4*A*, there was some enhancement of killing of cells coated with chimeric antibody alone at the 50:1 effector/target ratio, suggesting some ADCC activity. However, the 660 TIL line does not contain detectable numbers of cells expressing Fc receptors as shown by immunofluorescence analyses (unpublished data).

The ability to combine the targeting of a tumor-specific antibody together with a potent cytokine such as IL2 should prove useful in directing and localizing its effect at tumor sites. In this regard, tumors secreting either IL2, tumor necrosis factor, or granulocyte colony-stimulating factor after transfection with genes encoding these molecules are rejected upon transplantation into syngeneic animals due to the establishment of cellular immunity (21–23). Antibody targeting of cytokines may achieve this same end by stimulating a cytotoxic T-cell response and in this way augment the helper T-cell function that may be lacking in cancer patients.

The use of a whole antibody–IL2 fusion protein may have advantages over antibody fragment–IL2 fusion proteins, since additional effector functions (ADCC and CDC) will be targeted to the same site. As discussed earlier, experiments using peripheral blood cells from patients treated with IL2 have already shown that the ch14.18 antibody can mediate ADCC against tumor cells (12). In this case the primary effector cells are Fc receptor-positive ($CD16^+$) natural killer cells. The work presented here suggests that cytotoxic T cells can also be stimulated to kill autologous antigen-positive cells that have been coated with the antibody–IL2 fusion protein.

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Expression of Genetically Engineered Immunoconjugates of Lymphotoxin and a Chimeric Anti-Ganglioside GD2 Antibody

STEPHEN D. GILLIES, DELANO YOUNG, KIN-MING LO, SUSAN F. FOLEY, and RALPH A. REISFELD[1]

Research Department, Abbott Biotech, Needham Heights, Massachusetts 02194
[1]Research Institute of Scripps Clinic, La Jolla, California 92037

ABSTRACT

Human lymphotoxin was genetically conjugated to the constant region of a human gamma 1 immunoglobulin gene at the end of either the second (CH2-LT) or third (CH3-LT) constant region domain. The altered heavy chain constant regions were combined in a plasmid vector together with the variable regions of a mouse anti-ganglioside GD2 antibody 14.18 and the human kappa constant region. The resulting immunoconjugate constructs were expressed in transfected hybridoma cells and tested for both their antibody and lymphotoxin activities. The two constructs were assembled to varying degrees depending on whether the third heavy chain constant region was present. Both forms retained their ability to bind antigen and mediate ADCC but only CH3-LT was able to mediate the lysis of melanoma target cells in the presence of human complement. Lymphotoxin activity, as defined in a cytolytic assay with mouse fibroblasts, was found to increase significantly as a function of heavy chain assembly and to be equivalent to unconjugated lymphotoxin. Neither of the constructs were cytotoxic for antigen-bearing melanoma cells that are normally resistant to lymphotoxin and tumor necrosis factor α. Such immunoconjugates may prove useful in targeting cytokines to the site of antigen-bearing cells in vivo. In this case, as a means of eliciting an inflammatory response at the site of a solid tumor.

INTRODUCTION

Tumor necrosis factor (TNFα) and lymphotoxin (LT or TNFβ) were first identified on the basis of their ability to directly kill certain tumors, however, many other biological activities are now attributed to these closely related cytokines. These include effects on a variety of cell types such as the induction of histocompatibility antigens and adhesion receptors as well as those resulting in inflammation, vascular permeability changes and mononuclear cell infiltration (1-3). The very short half-life of both TNFα and LT ensures that these inflammatory reactions do not occur systemically but only at the sites of release from TNF-producing cells.

This ability to elicit a localized inflammatory response may prove useful in a combined immunotherapeutic approach to the treatment of solid tumors, together with a monoclonal antibody or antibody conjugate. For example, if it were possible to specifically deliver either TNFα or LT to the tumor site, a local inflammatory response might lead to an influx of effector cells such as natural killer cells, large granular lymphocytes, and eosinophils, i.e. the very cells that are needed for antibody-dependent cellular cytotoxicity (ADCC) activity. The increased vascular permeability may also allow more antibody to reach the tumor.

We have been studying the feasibility of genetically linking LT to the heavy (H) chain of the chimeric mouse/human immunoglobulin (Ig) ch14.18 with anti-tumor

specificity as a means of localizing LT at the sit f a human melanoma tumor stablished in immmod ficient mice. In this case the specificity of the mouse variable (V) regions is f r th disialoganglioside GD2 present on many tumors of neuroectod rmal origin such as melanoma and neuroblastoma (4). W have alr ady shown that this humanized antibody has potent biological effector functions in vitro and localizes in human melanoma xenografts in athymic nude mice (5).

One potential problem with expressing LT as a fusion protein to an Ig H chain is that the native LT molecule exists in solution as a trimer and binds more efficiently to its receptor in this form (6). Thus, it seems unlikely that trimerization could still occur when LT is attached to an H chain via its amino terminus and is assembled into an intact Ig molecule containing two paired H chain fusion polypeptides. Secondly, the ability of the fused LT to bind to its receptor may be severely compromised if a free amino terminus is required for receptor binding activity. In fact, it has been postulated that the amino and carboxy-termini of TNF (and by analogy, LT) together form a structure that is required for receptor interaction (7).

We have examined the fusion of LT to the gamma 1 H chain constant (C) region at two positions: at the end of the second (CH2) or third (CH3) domain. The resulting Ig/LT conjugates were expressed in transfected hybridoma cells and tested for chain assembly, antigen binding, antibody effector function and LT activity as defined by the ability to kill a sensitive mouse fibroblast cell line. The two constructs differ markedly in LT activity which is likely related to the extent of H-chain fusion protein assembly.

MATERIALS AND METHODS

Plasmid Construction

Plasmid pdHL2, containing the human Cγ1 heavy and kappa light chain gene sequences as well as insertion sites for V region cDNA cassettes (8), was used for the expression of Ig-LT fusion proteins. A LT cDNA was isolated from a human peripheral blood leukocyte library cloned in λgt10 (unpublished results). The sequence was identical to that reported in the literature (9). The cDNA was inserted into vector pDEM (8) as an XhoI fragment after first removing most of the 3′ untranslated region with Bal31 nuclease. The resulting plasmid, pDEM-LT (Figure 1), expresses (in transfected cells) a fusion mRNA with a 5′ untranslated sequence derived from the metallothionein (MT) promoter, the LT coding sequence and a 3′ untranslated sequence and polyA addition signal from the mouse Cκ gene. Fusion protein-encoding vectors were constructed by ligating HindIII to TaqI (CH2-LT) or HindIII to NsiI (CH3-LT) fragments of the human Cγ1 gene to HindIII and PvuII digested pDEM-LT using synthetic DNA linkers (Figure 1). These linkers (5′-CGAAGAAAACCATCTCCAAA/CTCCCTGGTGTTGGCCTCACACCTTCAG-3′ for CH2-LT and 5′-TGAGGCTCTGCACAACCACTACACGCAGAAGAGCCTCTCCCTGTCCCCGGGTAAA/CTCCCTGGTGTTGGCCTCACAC CTTCAG-3′) provide the protein coding sequence from the unique site (NsiI or TaqI) to the end of the heavy- chain domain (indicated by the slash) and join them to the amino terminus of the mature form of LT (up to the unique PvuII site). The linker for the CH3 fusion protein also includes a silent mutation that creates a SmaI site close to the end of the domain for future use in constructing fusion proteins. The DNA sequences at the junction of each construct were confirmed and each HindIII to EcoRI fragment was inserted into plasmid pdHL2-VCγ1κ(14.18). This plasmid contains the V cassettes for the ch14.18 anti-ganglioside GD2 antibody (8).

Cell Culture and Transfection

Sp2/0 Ag14 mouse hybridoma cells were maintained and transfected as described (10). Drug selection in methotrexate (MTX) was initiated 24 hr after transfection by adding an equal volume of medium containing MTX at 0.1 μM. Two additional feedings with selection medium were done at 3 day intervals. Tranfectants secreting human Ig determinants were identified by ELISA (10), grown in medium containing increasing concentrations of MTX, and subcloned by limiting dilution in medium containing MTX at 5 μM.

Purification and Characterization of Fusion Proteins

Proteins were biosythetically labeled by incubating transfected cells ($1x10^6$/mL) for 16 hr in growth medium containing ^{35}S-methionine (50

μCi/mL-Amersham). Culture supernatants were then clarified by centrifugation in a microcentrifuge and the labeled proteins were immunoprecipitated with polyclonal anti-human κ chain antis ra (Jackson Immunores arch) and protein A Sepharose (Repligen). Protein samples were boiled for 5 min. in gel sample buffer in the presence or absence of 2-mercaptoethanol and analyzed on 7% polyacrylamide-SDS gels. Labeled proteins were detected by fluorography (diphenyloxazole in DMSO) and autoradiography.

Unlabeled proteins were purified from spent suspension culture medium by either immunoaffinity chromatography with a monoclonal anti-human κ antibody for the CH2-LT protein or by protein A Sepharose chromatography for the CH3-LT protein. All materials were concentrated by membrane dialysis into PBS. An alternative procedure for purification of the CH3-LT protein was developed to prevent the loss of LT activity during elution from the protein A column. Spent culture media was diluted with three volumes of 10 mM sodium phosphate buffer (pH 6.5) and loaded onto a Bakerbond AbX (J.T. Baker) column at room temperature. The column was washed with 10 mM sodium phosphate buffer until the absorbance returned to baseline and then with PBS, pH 6.5 (150 mM NaCl, 10 mM sodium phosphate, pH 6.5). The CH3-LT protein was eluted with 150 mM NaCl, 50 mM sodium phosphate, pH 6.5.

The antigen binding activity of the Ig-LT proteins was measured as described (8) and LT activity was determined in the cytolytic or cytostatic assay (11) utilizing the 1591-RE3.5(24T2.5) subclone of the mouse L929 cell line (generously provided by Dr. H. Schreiber, University of Chicago). Cells were seeded into 96-well plates at 4 x 10^4 cells per well, with (cytolytic) or without (cytostatic) mitomycin C (2 μg/mL), and 10 μL of the test sample was added after 24 hr. Cells were stained either 24 or 48 hr later (see figure legends) with crystal violet and the amounts of dye retained in the wells were compared to those of untreated wells and those receiving the LT standard (R & D Systems). The same assay was also carried out with the GD2-bearing human melanoma line M21, originally provided by D.L. Morton, University of California, Los Angeles. The latter cell line was also used for measuring CDC and ADCC activity as described earlier (12).

RESULTS

Construction and Expression of Ig/LT Conjugates

The first Ig/LT constructs were made by directly fusing the cDNA sequence encoding the mature form of LT to the hinge region exon of the human Cγ1 gene. This gene fusion was then combined in a vector together with the V regions of murine antibody 14.18 (4) and the human Cκ gene. Following expression in transfected Sp2/0 cells, it was found that this construct encoded a Fab-like protein with LT fused to the carboxy-terminus. This protein did not exhibit any antigen binding activity and was not studied further (data not shown). A similar lack of antigen binding was obtained when we directly expressed Fab with these same mouse V regions (12) or produced them by proteolytic digestion of antibody. The LT sequence was then attached to the end of either the CH2 or CH3 exons of the human Cγ1 gene (Figure 1) with the appropriate synthetic linkers. These constructs were then expressed and tested for antigen binding activity and Ig chain assembly. Both constructs retained antigen binding when measured in a competitive antigen binding ELISA (see below) but differed greatly in assembly of the H chain fusion proteins. Cells expressing these constructs were labeled with ^{35}S-methionine and the secreted proteins were analyzed by SDS-PAGE in the presence or absence of reducing agent. As seen in Figure 2, the CH2-LT construct was expressed as a mixture of whole (approximately 180 Kd) and half (90Kd) molecules. The CH3-LT fusion protein, on the other hand, consisted entirely of fully assembled molecules. This result is not surprising since the CH3 domain is most responsible for Ig chain assembly (13). The reason a portion of the CH2-LT did assemble, i.e. formed disulfide bonds in the hinge domain of the antibody, is likely due to the dimerization of the carboxy-terminal LT domains, since similar constructs containing cytokines that do not normally dimerize (e.g. IL-2) do not assemble their H-chains (manuscript in preparation).

Biological Activity of Ig/LT Conjugates

The LT activities of the CH2-LT and CH3-LT conjugates wer compared in the

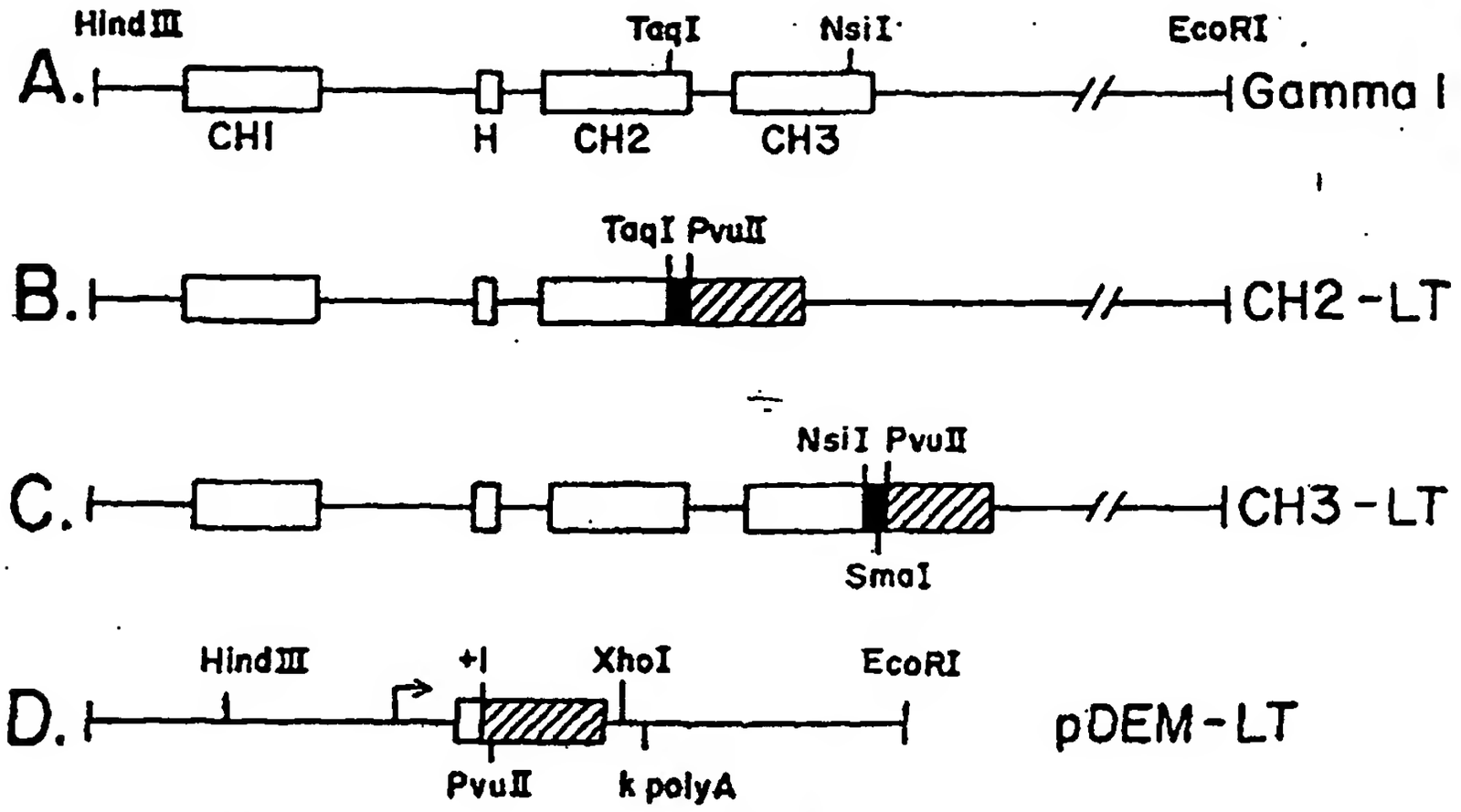

FIGURE 1. Construction of fusion proteins between LT and the human Ig H chain. (A) Restriction map of a human Cγ1 gene fragment cloned in plasmid pBR322. (B) The Cγ1 gene fused to LT at the end of the CH2 domain. (C) The Cγ1 gene fused to LT at the end of the CH3 domain. (D) The cDNA encoding LT cloned in expression vector pDEM including promoter (arrow), the natural leader peptide of LT (open box), the first residue of the mature protein (+1) and mouse κ L-chain poly A and 3'untranslated sequence. Open boxes represent protein coding regions of Cγ1 in A-C; black boxes represent synthetic linkers used to join the protein coding sequences; and striped boxes represent LT coding sequences.

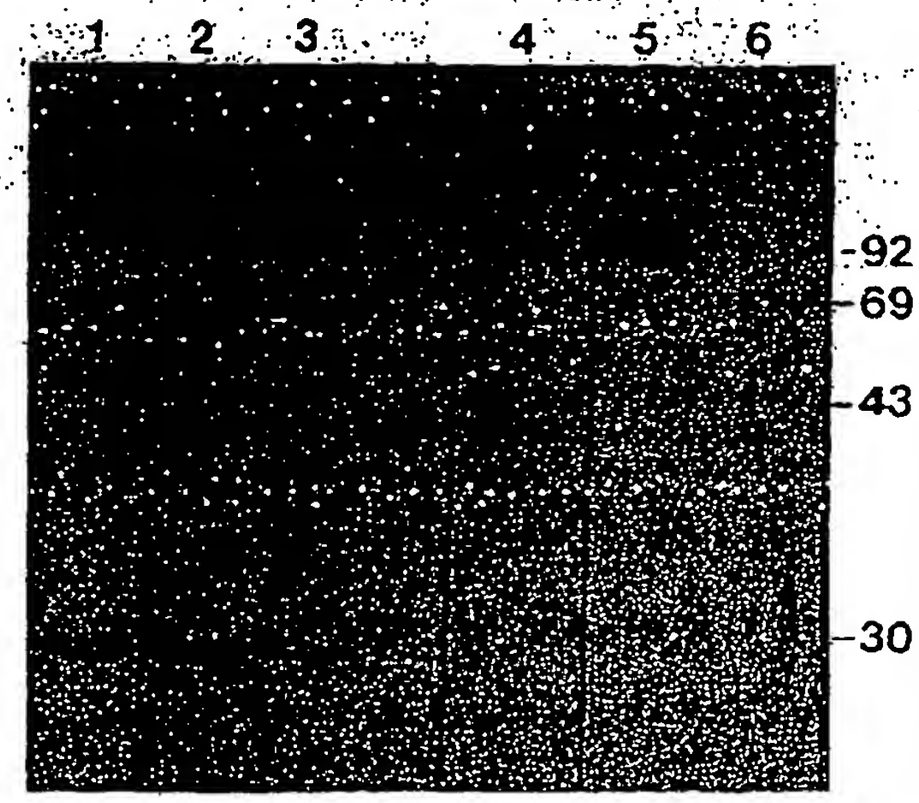

FIGURE 2. Analysis of fusion protein chain assembly. Cells expressing ch14.18 (lanes 1 and 4), CH2-LT (lanes 2 and 5), and CH3-LT (lanes 3 and 6) were labeled with ^{35}S-methionine and secreted proteins were precipitated with anti-human κ antiserum and protein A and analyzed on an SDS gel either reduced (lanes 1-3) or unreduced (lanes 4-6). The position of stained marker proteins and their apparent molecular weights are indicated. The dried gel was exposed to film for either 4 hr (lanes 1 and 4) or 18 hr.

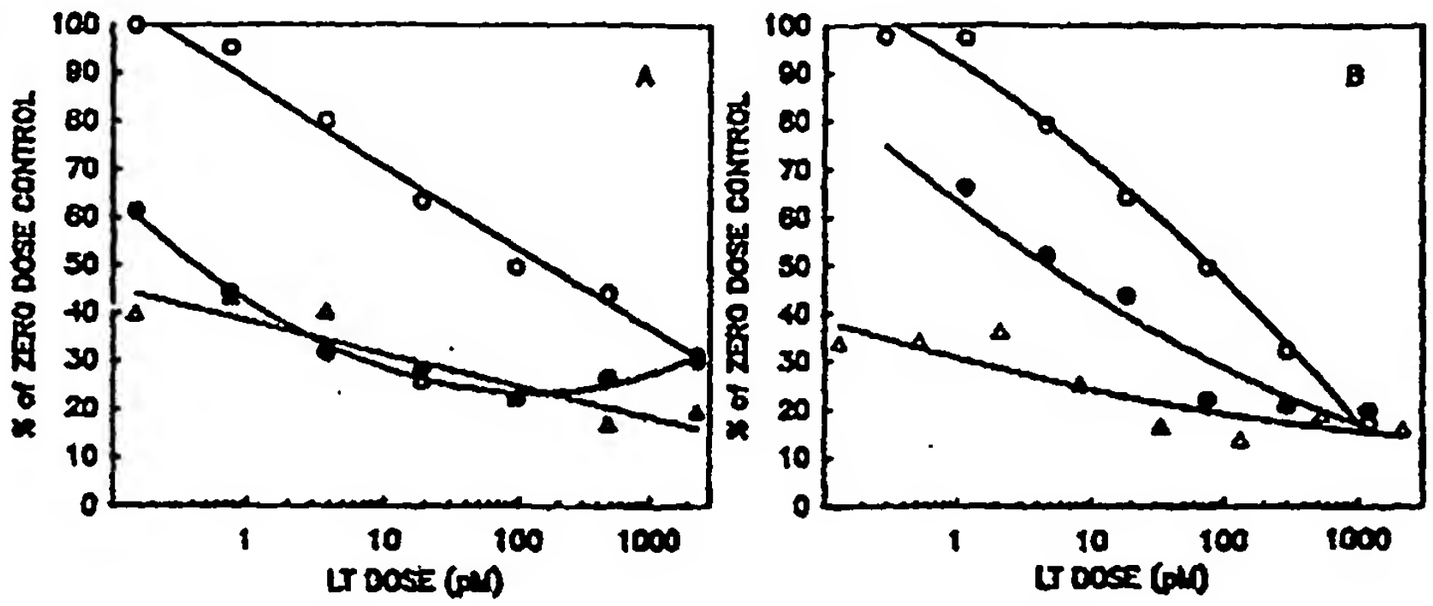

FIGURE 3. Comparison of LT cytolytic activities for native LT (δ—δ), and CH2-LT (o—o) or CH3-LT (•—•) immunoconjugates. A sensitive clone of the mouse fibroblast line 929 was used in the 1-day assay with mitomycin C. Relative cell survival was quantitated by staining with crystal violet and measuring the absorbance at 630 nm. (A) Culture supernants from transfected cells were assayed after first quantitating the conjugates by ELISA. (B) Purified proteins were assayed following protein A Sepharose or immunoaffinity chromatography. All concentrations were normalized to the mass contributed by LT.

standard cytolytic assay (11) with a mouse L929 subclone. This assay measures the ability of the conjugate to bind to the TNF/LT receptor and trigger the active cell killing process in this cell line. When crude preparations (culture supernatants) were compared (Figure 3A), CH3-LT was found to be much more active (nearly 100 fold by this assay) than CH2-LT and exhibited approximately the same specific activity per mole as the LT standard. This higher activity of CH3-LT is likely due to the increased proportion of fully assembled H-chain fusion proteins. Thus, the presence of the CH3 exon in the construct allows the H-chains to associate more efficiently, perhaps positioning the LT domains in a manner that allows for dimerization and, as a consequence, more LT receptor binding.

When purified preparations were compared, the difference in activities between CH2-LT and CH3-LT was still maintained but the activity of the conjugates, especially CH3-LT, was greatly reduced compared to the LT control (Figure 3B). Since both proteins had been purified by using elution steps at acidic pH (<pH 4), we examined the pH sensitivity of the culture supernatants and found the LT activity to be very acid labile (data not shown).

An alternative purification scheme was developed in which the pH was not reduced below 6.5, and material from this preparation was compared to that purified by protein A, the original starting material, and the LT standard. The results of the LT cytostatic assay, in the absence of mitomycin C, are shown in Figure 4 and demonstrate that full LT activity can be maintained during purification provided low pH is avoided. This assay was used to give a better dose response for the LT control and to demonstrate that the relationship between CH2-LT and CH3-LT is consistent for both assay systems. The same results were obtained in the cytolytic assay (not shown). Clearly, full activity (as measured by this assay) can be maintained when LT is fused to an Ig H chain. The fact that the LT amino terminus is covalently bound to the carboxy-terminus of the antibody apparently does not prevent LT receptor binding or the steps subsequent to binding that are required for activating the cell killing process. However, it should be pointed out that although the CH3-LT conjugate was generally as active as LT, the activity of the conjugate at extremely low concentrations was somewhat reduced.

Ig-LT Immunoconjugates do not Kill LT-resistant Antigen-bearing Tumor Cells

The cytotoxicity of the CH3-LT conjugate was tested against M21 human melanoma cells bearing a very high number of GD2 binding sites for the ch14.18 antibody. Although these cells are not sensitive to either LT or TNFα (unpublished results), it is possibl that the internalization of a greater number of LT molecules through the antigen/antibody route may r sult in cytotoxicity. The results of both the cytolytic and cytostatic assays (Figure 5A and 5B) indicat a

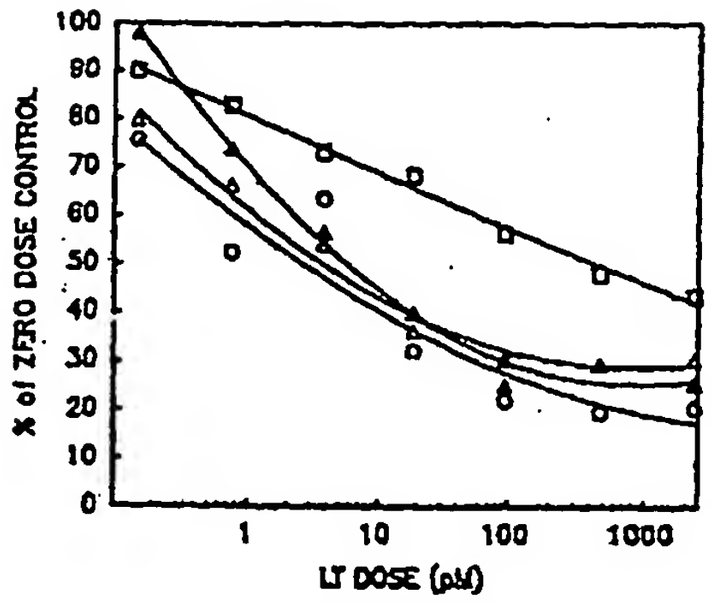

FIGURE 4. Effect of pH during purification on the cytostatic activity of CH3-LT. The activities of native LT (o—o), CH3-LT in culture supernatant (Δ—Δ), CH3-LT purified by protein A Sepharose chromatography (□—□) and CH3-LT purified at pH 6.5 (▲—▲) were compared in the cytostatic assay (in the absence of mitomycic C) using a mouse 929 subclone.

lack of M21 cell killing by either native LT or the CH3-LT conjugate. Although it has been shown that antibody bound to GD2 is internalized at a reasonable rate (14), we cannot exclude the possibility that the conjugate remains on the cell surface.

Antigen Binding and Effector Functions of Ig/LT Conjugates

The antigen binding activity of the conjugates was measured on antigen-coated plates in either a direct binding or competition assay format. In the direct binding assay antigen binding activity was found to be much higher than that of the control ch14.18 antibody (data not shown). Since the source of the GD2 antigen was a crude membrane extract from neuroblastoma cells, it is possible that the TNF/LT receptor is present in the preparation and that binding of the conjugate through the LT domain is responsible for this increased activity. When antigen binding was measured in a competition assay, the conjugate was found to compete with the labeled ch14.18 antibody for antigen only slightly more efficiently than the unlabeled ch14.18 antibody (Figure 6).

The effector functions of the Ig/LT conjugates were then tested by comparing their ability to mediate the lysis of GD2 antigen-bearing human melanoma cells by

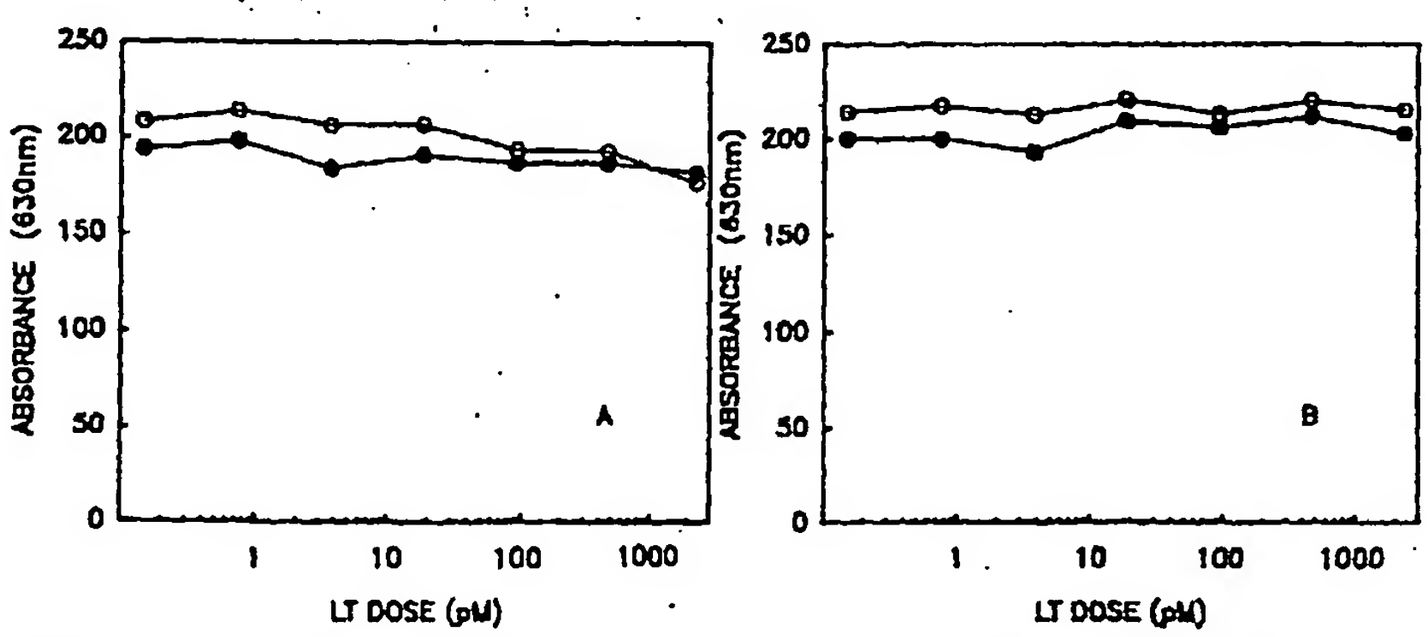

FIGURE 5. Cytolytic and cytostatic activities of LT and CH3-LT with GD2-positive M21 human melanoma cells. M21 cells were seeded in 96-well plates in the presence (A) or absenc (B) of mitomycin C and dilutions of LT (o—o) or CH3-LT (●—●) were added. Relativ cell growth was measured by staining wells with crystal violet after 48 hr and measuring the absorbance at 630 nm.

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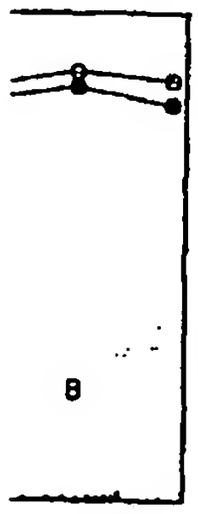

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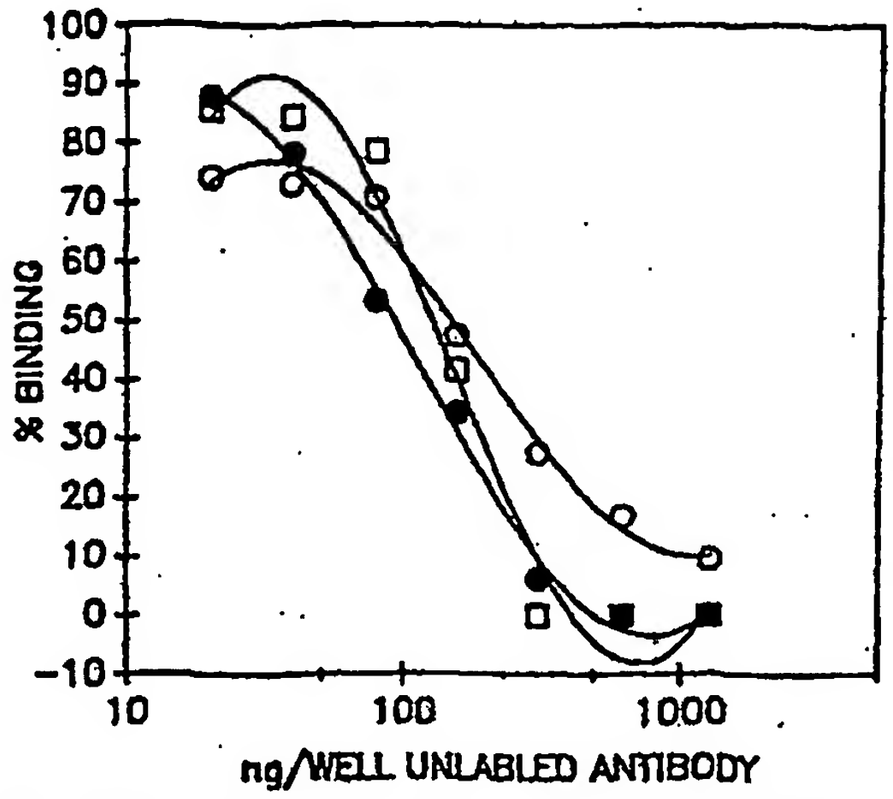

FIGURE 6. Antigen binding activity of Ig/LT immunoconjugates. Relative binding was determined in a competitive antigen binding assay using ch14.18 antibody conjugated to HRP as tracer and either unlabeled ch14.18 (o—o), CH2-LT (●—●) or CH3-LT (□—□) as competitor. Incubation was at 4° for 18 hr.

human complement (CDC activity) to that of the ch14.18 antibody. As seen in Figure 7A, the CH3-LT conjugate mediates lysis at approximately the same concentrations as ch14.18 suggesting that the fusion of LT to the carboxy-terminus of the H chain did not affect complement fixation. The CH2-LT conjugate, on the other hand, did not mediate lysis at concentrations as high as 10 μg/ml. The ability of the CH3-LT conjugate to mediate antibody-dependent cellular cytotoxicity (ADCC) also was not significantly impaired by the addition of LT to the carboxy-terminus (Figure 7B). In contrast, the CH2-LT conjugate required higher concentrations to achieve a comparable degree of lysis of the

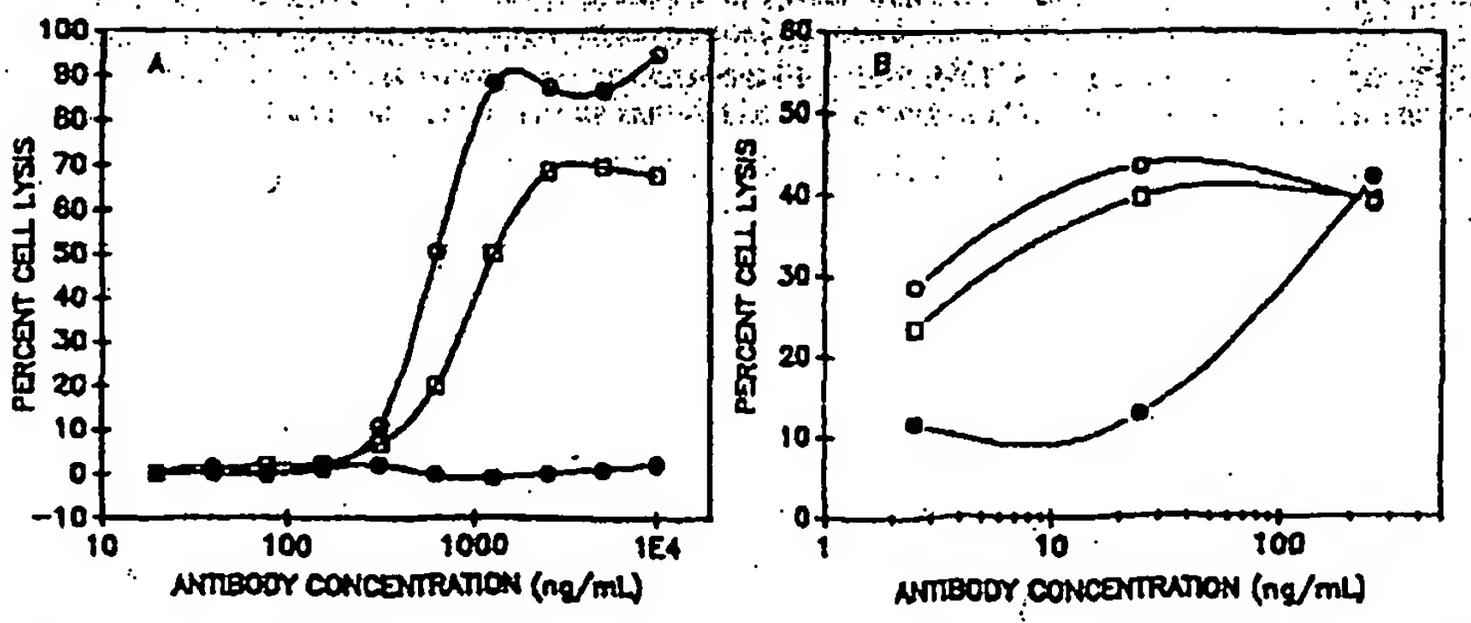

FIGURE 7. CDC and ADCC activities of ch14.18 and Ig/LT immunoconjugates. (A) The complement-dependent lysis of ^{51}Cr-labeled GD2-bearing M21 cells was assayed with the indicated amounts of ch14.18 antibody (o—o), CH2-LT (●—●) or CH3-LT (□—□) together with human complement. (B) The antibody-dependent lysis of ^{51}Cr-labeled M21 cells was assayed with the antibody or conjugates as above and freshly prepared PBLs at an effector to target ratio of 200:1. Both assays were carri d out for 4 hr at 37°. Th percent of specific lysis was calculated by subtracting the spontaneously released counts and dividing the corr cted experimental values by the total counts obtained by deterg nt lysis and multiplying by 100.

antigen-bearing melanoma target cells. Since only a fraction of the CH2-LT preparation was assembled into complete antibody molecules, it is possible that the assembled molecules are fully active in mediating ADCC but that higher concentrations are required to compensate for the fraction of inactive molecules.

DISCUSSION

The fusion of unrelated polypeptides by genetic engineering can often result in useful proteins of therapeutic value, especially when the desired biological functions of the individual proteins are fully maintained in the final product. The fusion of protein domains to the carboxy-termini of Ig H chains or H-chain fragments, as described in this report, can have unexpected consequences for the activities of both the protein to be fused and the antibody, particularly as far as antigen binding, assembly and effector functions are concerned.

We have shown in this report that it is possible to combine the antigen binding activity of an anti-tumor cell antibody with the potent biological activity of a cytokine. The most active conjugate, in terms of both antibody effector function and LT activity, was one in which LT was added to the end of the complete antibody (CH3-LT) instead of one in which LT replaces the third H-chain domain (CH3). The presence of the CH3 exon in the immunoconjugate results in complete H-chain assembly and, as a consequence, higher LT and effector activities. Although the assembly of H chains may likely result in LT dimerization, this possibility requires further examination by cross-linking studies. Clearly, the degree of H-chain assembly is not the only factor in the biological activity of the conjugates. CH2-LT and CH3-LT preparations differ by as much as 100-fold by activity but only by 2 to 3-fold in terms of assembly. The conformations of the LT portions of the dimerized conjugates must differ significantly to account for this discrepancy.

Our results also suggest that a free amino terminus is not necessary for LT binding to its receptor since in the highly active CH3-LT construct, the amino terminus of the LT domain is peptide bonded to the Ig H chain. This would agree with the results of Feng, et al., (15) who found that a fusion of LT to the carboxyterminal end of interferon-γ had demonstrable LT activity. However, we cannot rule out the possibility that the conjugate is proteolytically cleaved during the assay period and releases a free LT protein that binds to the receptor. In fact, we have found that plasmin treatment of the two conjugates neatly cleaves the Ig and LT molecules at, or very near, their junction and releases active LT while leaving the chimeric antibody intact (data not shown).

The antigen binding and effector functions of the chimeric mouse/human anti-GD2 antibody used in this study were also examined in order to assess whether the addition of LT to the carboxy-termini of the H chains would alter the antibody molecule and thereby change its ability to interact with its various ligands. Although some changes in antigen binding were noted in direct antigen binding assays, the magnitude of these changes is less apparent from competitive binding assay data. There is precedence for alterations in antigen binding activity as a consequence of modifying the Fc portion of an antibody. In fact, we have shown that deletion of the CH2 domain of the same ch14.18 antibody used in the present study dramatically increases its antigen binding capacity (12). We have also found that the fusion of other cytokines to either the CH2 or CH3 domains affects antigen binding either positively or negatively, depending on the particular protein (unpublished results). The two findings may have in common the fact that the interactions between Fab and the CH2 domain (which have been demonstrated by crystallographic studies) are disrupted, either by the deletion of CH2 or by the addition of another protein domain which may interact more strongly with CH2. In either case, the level of antigen binding may be proportional to the degree to which the Fab/CH2 interactions are disrupted, allowing for free movement of the Fab arms for contacting antigen. As discussed above, the Fab fragment of the 14.18 antibody and the Fab-LT conjugate do not bind antigen even though they lack the CH2 domain. This may be a specific property of this antigen/antibody combination since similar constructs made with the V regions of another antibody (B72.3) bind antigen. The difference between the two systems may reflect the need for bi-valency in binding GD2.

We have demonstrated that an antibody can be genetically fused to a cytokine without the loss of antig n binding activity and effector functions of the antibody, or the receptor binding and biological activity of LT. The ability of

such a conjugate to target LT to tumors in vivo, e.g. in tumor-bearing athymic mice, remains to be determined. While the CH3-LT conjugate may be useful for delivering LT activity to the tumor site and eliciting an inflammatory response, it probably not useful as a directly cytotoxic molecule. We have shown that the conjugate, with specificity for a highly abundant and internalized surface molecule (GD2), does not render an antigen positive, LT-resistant tumor cell sensitive to LT-mediated killing. This result is not surprising in light of recent evidence showing that TNF (and presumably LT) does not have to enter a cell in order to kill it (16). Thus, the mechanism of LT/TNF killing is likely due to specific receptor signalling rather than general uptake into the cell.

The fact that the conjugate does not directly kill the GD2-bearing tumor cells used in the present study should make it easier to interpret studies comparing the anti-tumor activities of the conjugate and chimeric antibodies. Further studies should also include a comparison of the present conjugate with one prepared using the coding sequence of TNFα. The structural similarities of LT and TNFα (1) suggest that it is possible to prepare active conjugates with this cytokine, thus making it possible to test the ability of each to elicit or enhance an anti-tumor response in vivo.

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Address correspondence to: Dr. Stephen D. Gillies,
Abbott Biotech, 119 Fourth Ave., Needham Heights, MA 02194
Telephone: (617)-449-6002 x2203.

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5

Effect of a Chimeric Anti-Ganglioside G_{D2} Antibody on Cell-mediated Lysis of Human Neuroblastoma Cells[1]

Edward Barker, Barbara M. Mueller, Rupert Handgretinger, Michael Herter, Alice L. Yu, and Ralph A. Reisfeld[2]

Department of Immunology, Research Institute of Scripps Clinic, La Jolla, California 92037 [E. B., B. M. M., R. A. R.]; Department of Hematology, Children's Hospital, University of Tübingen, D-7400 Tübingen, Federal Republic of Germany [R. H., M. H.]; and Department of Pediatrics, University of California Medical Center, San Diego, California 92103 [A. L. Y.]

ABSTRACT

An anti-G_{D2} ganglioside human/mouse chimeric monoclonal antibody, ch14.18, like its murine counterpart, 14.G2a, was shown to bind to human neuroblastoma cells. This chimeric antibody proved to be more effective than 14.G2a in mediating the lysis of neuroblastoma cells with human effector cells, such as granulocytes and natural killer cells within the peripheral blood mononuclear cell population. A comparison of these two effector cell populations isolated from the same donor revealed granulocytes to be more effective than peripheral blood mononuclear cells in lysing neuroblastoma cells, which were coated with monoclonal antibody ch14.18. Addition of recombinant human granulocyte-macrophage colony-stimulatory factor increased ch14.18-mediated lysis of neuroblastoma cells by granulocytes but not by peripheral blood mononuclear cells. In fact, granulocytes were effective in mediating lysis of neuroblastoma cells coated with ch14.18 irrespective of whether they were obtained from normal adults or from neuroblastoma patients.

INTRODUCTION

Neuroblastoma is one of the most common solid tumors of children that generally has a poor prognosis, since at the time of diagnosis, the majority of patients have widespread disease (1–3). The treatment available for such patients consists of surgical removal of solid tumor(s) followed by massive chemotherapy with or without radiotherapy and/or bone marrow reconstitution (4–7). Such treatment protocols can result in complete remission in some patients; however, in many such cases, the relapse rate is relatively high. It is apparent from these findings that additional adjuvant treatment modalities are needed to improve the outcome of this neoplastic disease.

In this regard, the use of mAbs[3] directed to neuroblastoma-associated antigen has been pursued both in the diagnosis and treatment of neuroblastoma (8–11). One such antigen targeted by mAbs that is frequently expressed on neuroblastoma is disialoganglioside G_{D2}, a glycolipid which is expressed on tumor cells of neuroectodermal origin (10, 12, 13). The G_{D2} antigen is ideal for mAb-mediated therapy of neuroblastoma, since it is expressed at high density on almost all neuroblastoma cells, is poorly expressed or absent from most normal tissue, and does not modulate its expression upon mAb binding.

Several mouse monoclonal anti-G_{D2} antibodies were reported to effectively suppress the growth of tumors of neuroectodermal origin in athymic (*nu/nu*) mice (14, 15), and one was shown to cause partial, as well as complete, regression in Phase I clinical trials (8, 16). The mechanisms believed to be involved in the ability of anti-G_{D2} mAb to suppress the growth of neuroectodermal tumors *in vivo* are antibody-mediated cellular cytotoxicity and/or complement-dependent cytotoxicity.

There are, however, several drawbacks in using murine monoclonal antibodies for therapeutic purposes in humans. First, such antibodies induce a human anti-mouse antibody response (17, 18); second, the half-life of murine mAbs in the circulation is relatively short compared to human immunoglobulin (19); and third, the Fc portion of murine antibody may not elicit ADCC or complement-dependent cytotoxicity as effectively as the Fc portion of a human antibody (20). To overcome these problems, recombinant DNA technologies have been applied to develop human/mouse chimeric monoclonal antibodies, containing the variable region of the murine antibody and the constant region of human immunoglobulin heavy and light chains (21).

The monoclonal antibody ch14.18 is an example of such a human/mouse chimeric antibody that is directed to ganglioside G_{D2} and which was developed by combining the complementary DNA sequences encoding the constant portion of human $\gamma 1$ heavy chain and κ light chain with those encoding the variable portions of immunoglobulin from murine hybridoma 14.18 (22). This chimeric antibody bound to melanoma tumor cells *in vitro* and reacted with melanoma tumors in athymic nude mice equally well as the equivalent mouse antibody. In addition, while ch14.18 was equal to its murine equivalent in mediating complement-dependent lysis of melanoma cells, it proved considerably better in mediating ADCC with peripheral blood mononuclear effector cells (23).

Here, we report the effects of ch14.18, as compared to its murine equivalent 14.G2a in ADCC of neuroblastoma cells by PBMC from normal donors, as well as from neuroblastoma patients. In addition, we established the population(s) of cells found in the peripheral blood that are primarily involved in ch14.18-mediated lysis of neuroblastoma cells. This study also served to determine whether cytokines affect the magnitude of lysis of neuroblastoma cells by peripheral blood cells in the presence of the human/mouse chimeric antibody.

MATERIALS AND METHODS

Cell Lines and Monoclonal Antibodies. The human neuroblastoma cell lines NMB-7 and IMR-32 were a gift from Dr. S. K. Liao (McMaster University, Hamilton, Ontario, Canada). These cells were grown in RPMI 1640 medium (Whittaker Bioproducts, Walkersville, MD) and supplemented with 10% fetal bovine serum (Whittaker Bioproducts). The adherent cell lines were detached with 0.5 mM EDTA (Sigma Chemical Co., St. Louis, MO), 0.02 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Fisher Chemicals, Fair Lawn, NJ), and 0.15 M NaCl (Mallinckrodt, Paris, KY), washed once, and then used as a single cell suspension.

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[2] To whom requests for reprints should be addressed, at Department of Immunology, IMM13, Research Institute of Scripps Clinic, 10666 N. Torrey Pines Road, La Jolla, CA 92037.

[3] The abbreviations used are: mAb, monoclonal antibody; GM-CSF, granulocyte-macrophage colony stimulatory factor; NK, natural killer; PBMC, peripheral blood mononuclear cell; ADCC, antibody-dependent cellular cytotoxicity; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; M-CSF, monocyte colony stimulatory factor; E:T, effector:target.

The hybridoma producing 14.G2a was established in our laboratory, as reported previously (15) and was produced in large amounts by BioTechnetics, San Diego, CA. The construction of the human/mouse chimeric antibody, ch14.18, has been described elsewhere (22).

Indirect Immunofluorescence. Neuroblastoma cells were incubated for 1 h at 4°C with 10 μg/ml of mAb. The cells were washed 3 times with PBS (Whittaker Bioproducts) containing 0.5% bovine serum albumin (Boehringer Mannheim Biochemicals, Indianapolis, IN) and 0.02% sodium azide (Mallinckrodt), followed by subsequent staining with either goat anti-human Fab FITC (for ch14.18) or goat anti-mouse IgG plus IgM-FITC for 1 h at 4°C. Both FITC-coupled antibodies were obtained from BMB, Indianapolis, IN. The cells were washed 3 times with PBS containing 0.5% bovine serum albumin and 0.02% sodium azide prior to analyzing an aliquot of 1×10^4 cells on a Fluorescent Activated Cell Sorter 400 flow cytometer (Becton Dickinson, Mountain View, CA).

Effector Cells. The source of effector cells was heparinized venous blood obtained from either healthy adult donors or from stage IV neuroblastoma patients at least 4 weeks after their last therapy. The whole blood was layered onto a Ficoll-Paque (Pharmacia, Uppsala, Sweden) gradient (1.077 g/ml) and centrifuged at 800 × g for 25 min. PBMC were collected at the interface between the Ficoll-Paque and the plasma. Subpopulations of PBMC from normal donors were isolated by adherence of PBMC to gelatin/fibronectin-coated flasks (24). The nonadherent cell preparation consisted of 65–80% $CD3^+$ cells, 5–10% $CD57^+$ cells, and ≤1% $CD14^+$ cells, as determined by microfluorometric analysis of 1×10^4 cells with fluorescein-conjugated antibodies (Becton Dickinson) directed to various cell surface markers. Adherent cells were obtained by treating PBMC, which were attached to the gelatin-fibronectin-coated flasks with EDTA. This adherent population was composed of ≥90% $CD14^+$ cells and a small population of <1% $CD3^+$ and <0.1% $CD57^+$ cells. NK cells were isolated by depleting T- and B-lymphocytes from nylon wool-nonadherent cells by an immunomagnetic method. To this end, nylon wool-nonadherent cells were first incubated with anti-CD3 and anti-CD19 antibodies (Becton Dickinson) for 30 min at 4°C, washed twice with PBS, and then further incubated under continuous shaking with sheep anti-mouse-coated immunobeads (Dynabeads; Dynal, Great Neck, NY) for 1 h at 4°C at a ratio of 20 beads/lymphocyte. Cells were then separated by placing them into a magnetic field (25). Granulocytes were isolated from pellets after fractionation of blood on a Ficoll gradient. Contaminating erythrocytes were removed by prior lysis of cell pellets with either distilled water or ammonium chloride. This cell preparation contained 95–99% granulocytes, as determined by microfluorometric analysis of an aliquot of 1×10^4 cells by indirect staining with the granulocyte-specific monoclonal antibody PMN-8C7 (Sigma Chemical Co.) as the primary antibody (26, 27).

Antibody-dependent Cellular Cytotoxicity. Tumor cells (5×10^5) were labeled for 2 h at 37°C with 0.5 mCi of sodium ^{51}Cr (10–35 mCi/ml, Amersham Corp., Arlington Heights, IL) in 0.5 ml RPMI 1640. After washing these cells 3 times, aliquots of $1\text{-}2 \times 10^3$ cells in a 25-μl volume were plated in 96-well U-bottomed microtiter plates (Corning Laboratory Sciences, Corning, NY). Human/mouse chimeric mAb ch14.18 or murine mAb 14.G2a were added each in a 50-μl volume to triplicate wells containing tumor cells. The concentration of antibodies ranged between 1 and 0.001 μg/ml. Effector cells were placed into microtiter wells in a total volume of 100 μl at effector:target cell ratios of 200:1, 100:1, 50:1, or 25:1. In assays in which either 10–100 ng/ml of human recombinant GM-CSF (a gift of BehringWerke, Marburg, Federal Republic of Germany) or 1000 units/ml of human recombinant M-CSF (Genzyme, Boston, MA) were admixed, the total volume of cells added was reduced to 50 μl, with the other 50-μl volume consisting of either the cytokines or media. Lysis of target cells mediated by effector cells alone was determined for each blood donor by incubating the tumor cells and the effector cells together in the absence of antibody. The plates were incubated for 4 h at 37°C and then centrifuged at 100 × g for 2 min. A 100-μl aliquot of each supernatant was harvested and analyzed for radioactivity in a Comp/Gamma counter (Pharmacia LKB Biotechnology, Gaithersburg, MD). Total ^{51}Cr release was measured by lysing the tumor cells with 10% sodium dodecyl sulfate. Spontaneous ^{51}Cr release was determined in wells that contained only tumor cells. Percentage of specific lysis was calculated as:

$$\frac{\text{Experimental } ^{51}\text{Cr release} - \text{mean spontaneous } ^{51}\text{Cr release}}{\text{Mean maximal } ^{51}\text{Cr release} - \text{mean spontaneous } ^{51}\text{Cr release}} \times 100$$

The data are presented as the mean percentage of specific release of three replicates. Although the percentage of specific ^{51}Cr release exerted by effector cells from various individuals varied from experiment to experiment, the pattern of the results remained consistent.

RESULTS

Binding of Human/Mouse Chimeric mAb ch14.18 to Surface of Neuroblastoma Tumor Cells. The NMB-7 neuroblastoma cell line used as a target cell throughout this study is known t express the ganglioside G_{D2} (14). Here, it was determined whether ch14.18 mAb could recognize G_{D2} and bind to the NMB-7 cell line. As a control, the same neuroblastoma cell line was tested for its ability to bind murine mAb 14.G2a. As shown in Fig. 1, both ch14.18 and 14.G2a stained all the NMB-7 cells, as determined by indirect immunofluorescence.

mAb ch14.18-mediated Lysis of Neuroblastoma Tumor Cells by PBMC. Here, we compared the ability of ch14.18 and its murine counterpart, 14.G2a, to mediate the lysis of neuroblastoma cells by PBMC. As shown in Fig. 2, ch14.18 was more effective in mediating the lysis of NMB-7 neuroblastoma cells by PBMC at an E:T of 100:1, since less ch14.18 was required than the murine mAb to achieve the same lytic effect. Proportionately, similar results were obtained at E:T ratios of

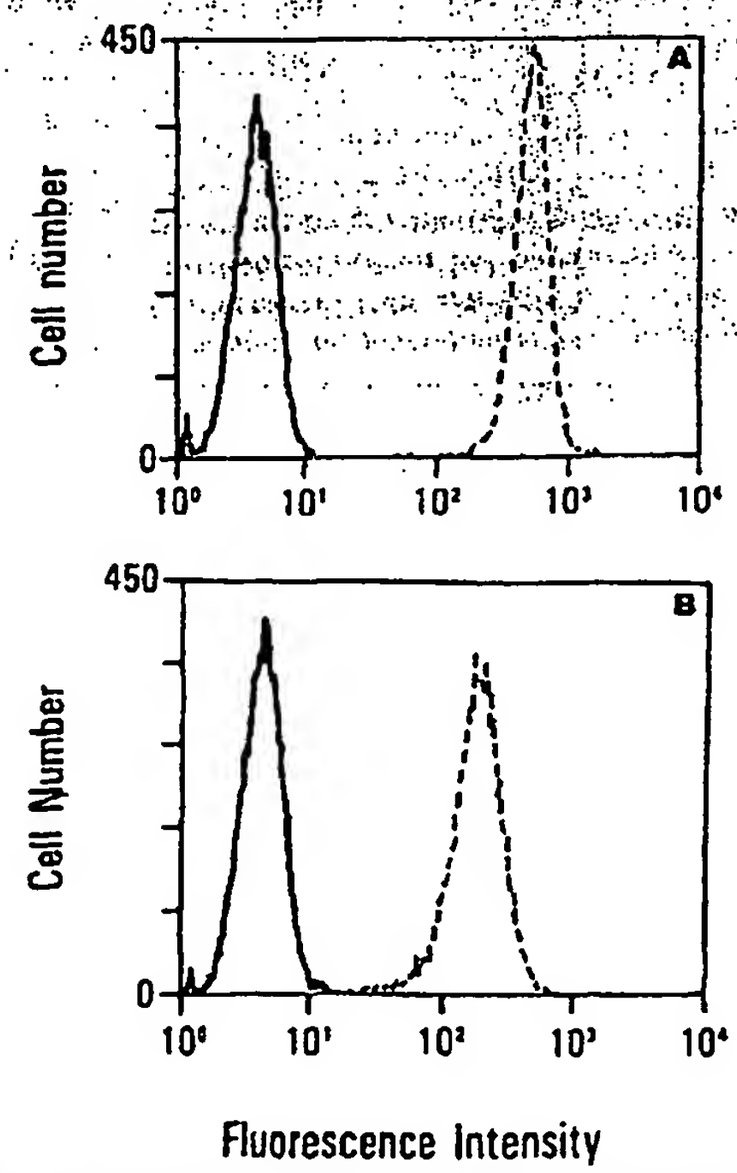

Fig. 1. Flow cytometric analysis of neuroblastoma cell line NMB-7 after staining of cells with murine mAb 14.G2a and goat anti-mouse-FITC (Fig. 1A) or human/mouse chimeric mAb ch14.18 and goat anti-human FITC (Fig. 1B). ——, staining with secondary antibody alone; ——, staining with primary and secondary antibody.

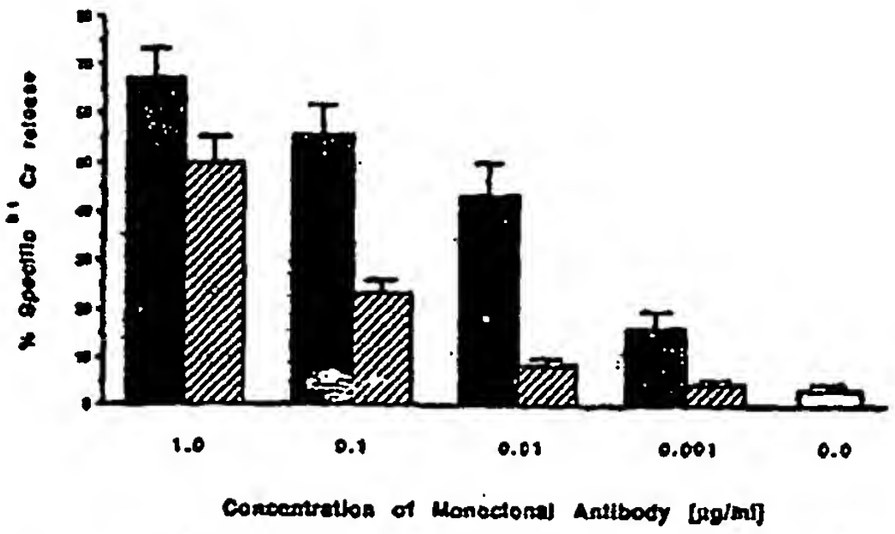

Fig. 2. Antibody-dependent cellular cytotoxicity mediated by PBMC against NMB-7 neuroblastoma tumor cells in the presence of either ch14.18 (■) or 14.G2a (▨) antibodies. Cytotoxicity was measured in a 4-h ^{51}Cr release assay at E:T of 100:1. □, level of lysis of NMB-7 cells mediated by PBMC in the absence of antibody.

Table 1 *Antibody-dependent cytotoxic activity of PBMC from neuroblastoma patients against IMR-32 neuroblastoma cells in the presence of either ch14.18 or 14.G2a antibodies*

Patient	Concentration of antibody (µg/ml)	% of specific ^{51}Cr release at an E:T of: 10:1	5:1	2.5:1	1.25:1
J. B.	0	0	0	0	0
J. B.	2.5	39[a]/20[b]	30/16	15/7	13/5
J. B.	0.04	50/10	34/6	25/3	12/0
J. B.	0.005	46/12	33/4	21/2	14/0
K. R.	0	22	5	2	0
K. R.	2.5	33/35	22/21	22/15	17/10
K. R.	0.04	45/38	35/25	25/12	20/6
K. R.	0.005	53/24	35/26	25/12	15/8
F. K.	0	18	4	0	0
F. K.	2.5	43/42	21/18	14/10	7/1
F. K.	0.04	57/48	30/21	20/10	9/4
	0.005	58/50	30/20	24/11	17/3

[a] Ch14.18 antibody.
[b] 14.G2a antibody.

200:1 and 50:1 (data not shown). This observation is not limited to the NMB-7 cell line, since LAN-1 and IMR-32 neuroblastoma cells were also lysed by PBMC more efficiently when mediated by ch14.18 rather than 14.G2a (data not shown).

The results depicted in Fig. 2 are from experiments that made use of PBMC isolated from normal adults. Additional studies were done to assess whether similar results could be achieved with PBMC from neuroblastoma patients. Table 1 summarizes the results that were obtained with PBMC isolated from the blood of 3 such patients that were tested individually. Overall, ch14.18 mediated the lysis of neuroblastoma tumor cells by PBMC from neuroblastoma patients more effectively than 14.G2a.

Extent of mAb ch14.18-mediated Lysis of Neuroblastoma Cells by Different Subpopulations of PBMC. In order to test the effect of different PBMC subpopulations on ADCC, these cells were separated based on their ability to adhere to fibronectin-coated surfaces (24). Adherent and nonadherent cells thus obtained were assayed for their ability to mediate the lysis of NMB-7 neuroblastoma cells with mAb ch14.18. As illustrated in Fig. 3, at E:T of 100:1, effective ch14.18-mediated lysis of NMB-7 cells was achieved with the nonadherent PBMC population and only marginal lysis was obtained with adherent PBMC. A similar pattern of results was observed at E:T of 50:1 and 25:1 with proportionately lower values (data not shown). The addition of M-CSF (1000 units/ml) to this assay did not increase the ability of adherent PBMC to lyse NMB-7 tumor cells in the presence of ch14.18 (data not shown). In ADCC experiments with enriched NK cells from healthy adult donors (>90% $CD56^+$ cells), ch14.18 was more effective than 14.G2a, especially at lower antibody concentrations of 5–2.5 mg/ml and lower E:T ratios of 2.5:1 to 1.25:1 (Table 2).

Comparison of ch14.18- and 14.G2a-mediated Lysis of Neuroblastoma Tumor Cells by Granulocytes and PBMC. Here, we wanted to determine whether granulocytes, as well as PBMC, can mediate the lysis of neuroblastoma with ch14.18 better than murine mAb 14.G2a. The data in Fig. 4 illustrate that whenever the concentration of antibody used in these experiments was high (>0.1 µg/ml), granulocytes were capable of mediating the lysis of neuroblastoma equally well with both mAbs. However, at antibody concentrations of <0.1 µg/ml, the ability of mAb ch14.18 to mediate the lysis of NMB-7 tumor cells by granulocytes exceeded that of its murine counterpart. Fig. 4 shows results obtained at E:T of 100:1. The same experiments were also done at E:T of 200:1 and 50:1, where essentially the same pattern of results was obtained (data not shown).

In an effort to assess whether ch14.18-mediated lysis of NMB-7 cells was more effective with granulocytes than PBMC, these effector cells were isolated from the blood of the same donors and compared for their ability to mediate tumor cell lysis in the presence of ch14.18. The data in Fig. 5 demonstrate that granulocytes are superior to PBMC in mediating ch14.18-directed lysis of NMB-7 cells, since it required less antibody for tumor cell lysis mediated by granulocytes than by PBMC. The data in Fig. 5 are from experiments done at an E:T ratio

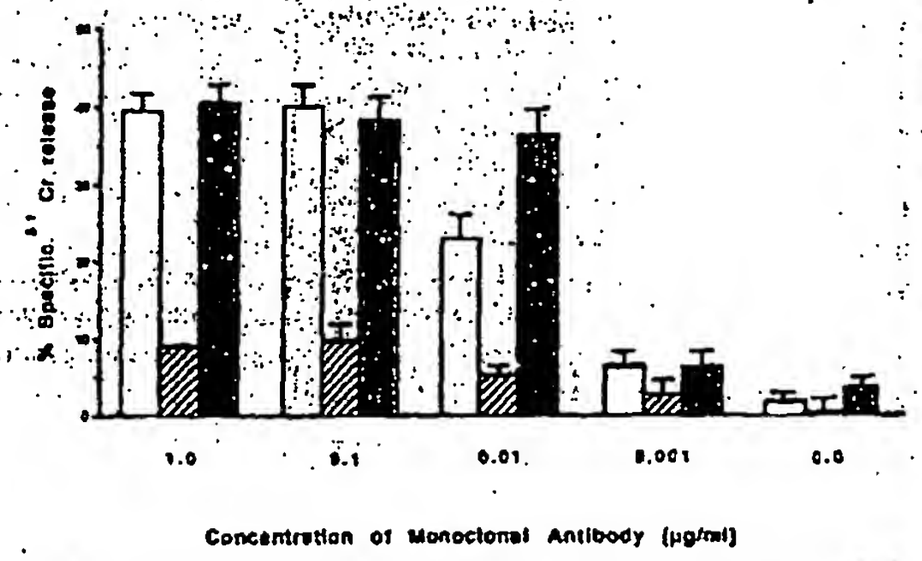

Fig. 3. Effect of adherent and nonadherent PBMC in mediating ch14.18 antibody-dependent cytotoxic activity against NMB-7 neuroblastoma tumor cells. Unfractionated PBMC (□), adherent PBMC (▨), and nonadherent PBMC (■) were tested for their ability to mediate ADCC activity. Cytotoxic activity was measured in a 4-h ^{51}Cr release assay at E:T of 100:1.

Table 2 *Antibody-dependent cytotoxic activity of enriched NK cells from normal donors against IMR-32 neuroblastoma cells in the presence of either ch14.18 or 14.Ga2 antibodies.*

Donor	Concentration of antibody (µg/ml)	% of specific ^{51}Cr release at an E:T of: 10:1	5:1	2.5:1	1.25:1
1	0	26	7	0	0
	2.5	77[a]/71[b]	62/54	18/16	7/4
	0.04	74/67	65/56	32/20	8/4
	0.005	75/51	59/39	28/11	9/2
	0.0025	70/48	52/32	16/5	5/0
2	0	40	25	19	7
	5.0	85/74	70/68	50/42	ND
	0.16	77/67	73/62	58/42	33/21
	0.04	77/57	73/45	54/27	33/15
	0.02	75/56	65/35	50/24	28/13
	0.01	79/41	61/41	49/23	28/12

[a] Ch14.18.
[b] 14.G2a.

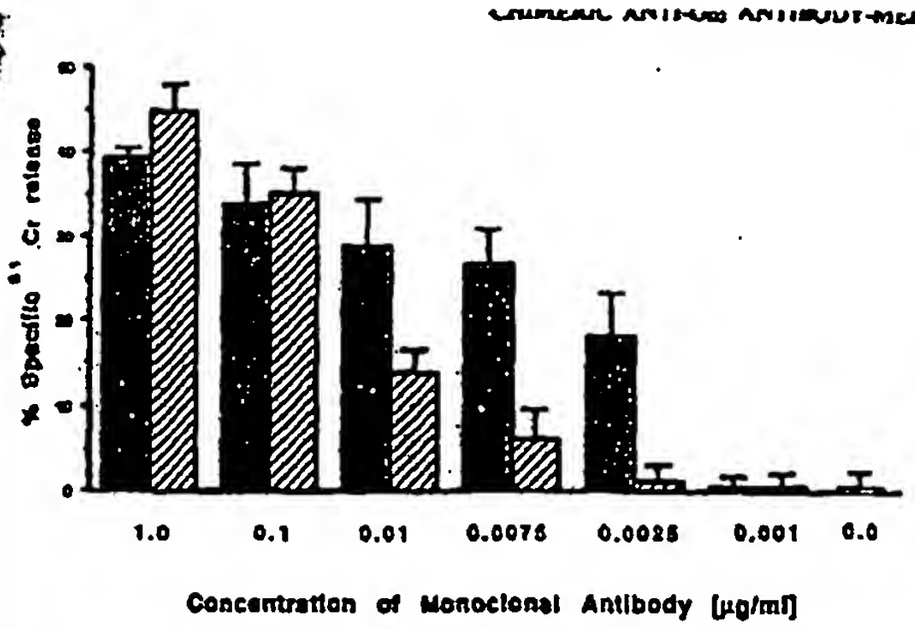

Fig. 4. Antibody-dependent cytotoxicity mediated by granulocytes against NMB-7 neuroblastoma tumor cells in the presence of either ch14.18 (■) or 14.G2a (▨) antibodies. Cytotoxicity was measured in a 4-h ^{51}Cr release assay at E:T of 100:1. □, level of lysis of NMB-7 cells mediated by granulocytes in the absence of antibody.

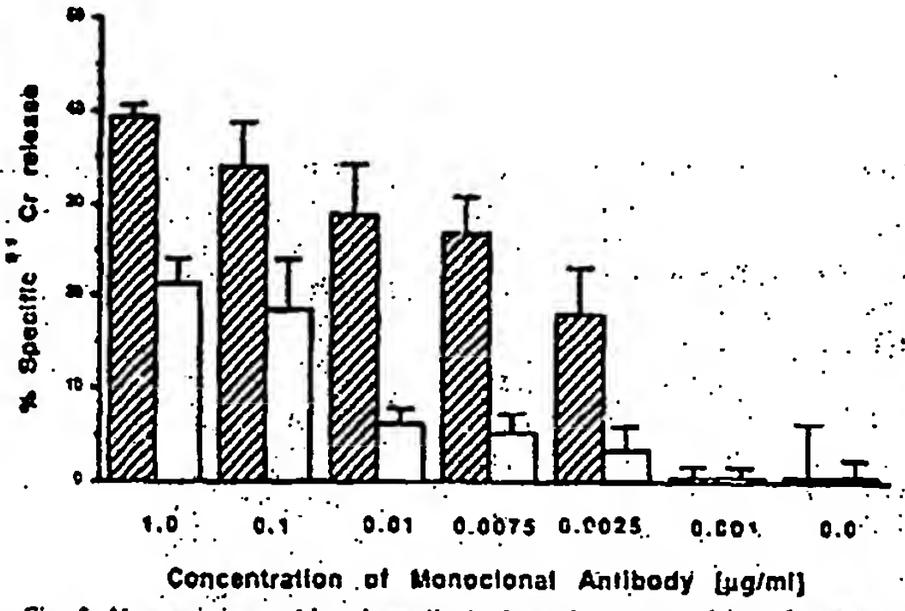

Fig. 5. Human/mouse chimeric antibody-dependent cytotoxicity of NMB-7 neuroblastoma tumor cell line mediated by either granulocytes (▨) or PBMC (□). Cytotoxicity was measured in a 4-h ^{51}Cr release assay at E:T of 100:1.

of 100:1. E:T ratios of 200:1 and 50:1 produced essentially the same pattern of results (data not shown).

In this study, we determined whether ch14.18-mediated lysis of NMB-7 by granulocytes could also be augmented by GM-CSF in a 4-h ^{51}Cr release assay at E:T of 50:1 (Fig. 6). At concentrations of 100 and 10 ng/ml of the cytokine, GM-CSF did indeed augment the ability of the granulocytes to mediate lysis of NMB-7 tumor cells with ch14.18. In contrast, another aliquot of the same batch of GM-CSF was not effective in enhancing ch14.18-mediated lysis of NMB-7 tumor cells by PBMC (data not shown).

Comparison of ch14.18-mediated Lysis of Neuroblastoma Tumor Cells by PBMC and Granulocytes from Neuroblastoma Patients. Experiments were performed to determine whether granulocytes from neuroblastoma patients are able to lyse NMB-7 neuroblastoma cells in the presence of ch14.18 equally well as granulocytes from normal adult donors. In addition, the extent of ch14.18-mediated lysis of NMB-7 by granulocytes from neuroblastoma patients was compared with that achieved by PBMC isolated from the same patient. These analyses were done at E:T ratios of 75:1 to 25:1 and at an antibody concentration of 1 μg/ml. As demonstrated in Fig. 7, similar to granulocytes from normal adults, granulocytes from neuroblastoma patients proved superior to PBMC in ch14.18-mediated lysis of NMB-7 tumor cells. In the presence of 100 ng/ml of GM-CSF, a further increase in ch14.18-mediated lysis by granulocytes was obtained, but only at low E:T ratios. In contrast, an aliquot of the same batch of GM-CSF failed to increase the ability of PBMC to lyse NMB-7 tumor cells in the presence of ch14.18. Taken together, these data indicate that in the presence of ch14.18, granulocytes from both neuroblastoma patients and normal adults have a greater ability to lyse NMB-7 neuroblastoma cells than PBMC isolated from the same individuals, and that the magnitude of this lysis is increased even further following stimulation of these effector cells with GM-CSF.

DISCUSSION

The human/mouse chimeric antibody ch14.18, like its murine counterpart, 14.G2a, binds to neuroblastoma tumor cells. This chimeric antibody also proved to be more efficient than 14.G2a in mediating the lysis of neuroblastoma tumor cells with such effector cells as NK cells within nonadherent PBMC population, as well as granulocytes. When these two effector cell populations were compared, granulocytes were far better than

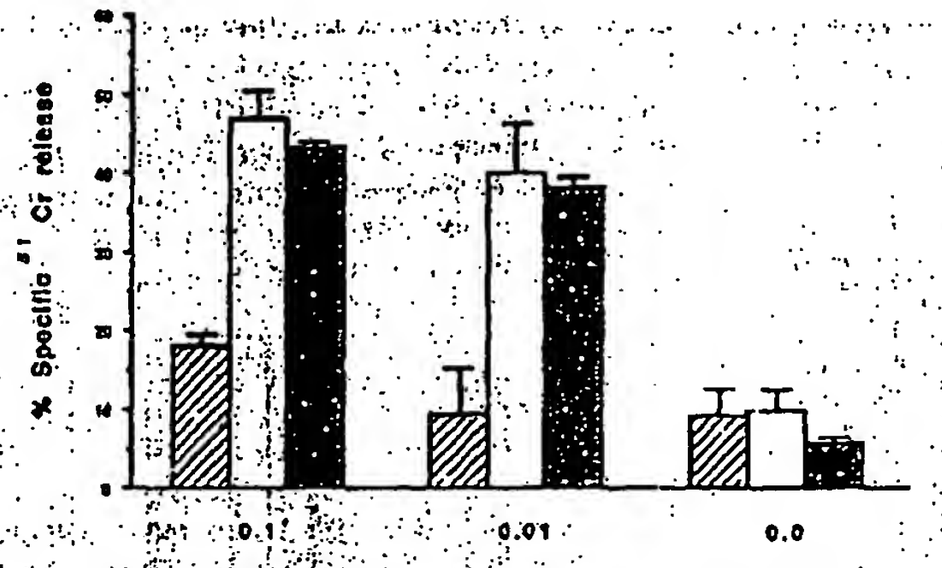

Fig. 6. Ch14.18-mediated lysis of NMB-7 neuroblastoma tumor cells by either granulocytes in the absence (▨) or presence of either 100 ng/ml (□) or 10 ng/ml (■) recombinant human GM-CSF. Cytotoxicity was measured in a 4-h ^{51}Cr release assay at E:T of 50:1.

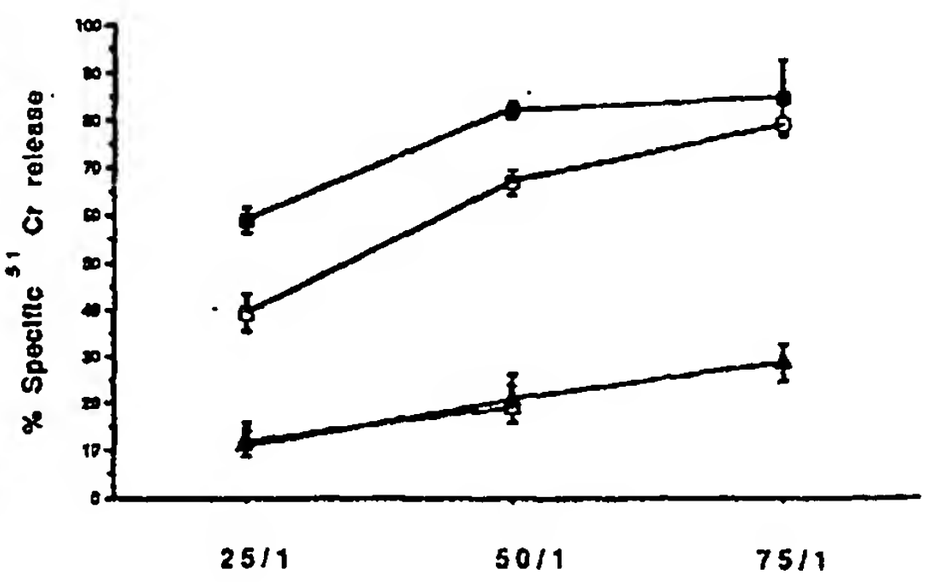

Fig. 7. Ch14.18-mediated lysis of NMB-7 neuroblastoma tumor cells by either granulocytes (□, ■) or PBMC (Δ, ▲) from the same neuroblastoma patient in the absence (□, Δ) or presence (■, ▲) of recombinant human GM-CSF (100 ng/ml). Cytotoxicity was measured in a 4-h ^{51}Cr release assay at various E:T ratios of 75:1, 50:1, and 25:1. The concentration of chimeric antibody used was 1 μg/ml.

PBMC in lysing neuroblastoma cells in the presence of ch14.18. Human recombinant GM-CSF further increased ch14.18-mediated lysis of neuroblastoma tumor cells by granulocytes, an effect which was not obtained with PBMC.

We were interested in determining the phenotype of the cells within the PBMC population, which are primarily responsible for ch14.18-mediated lysis of neuroblastoma cells. It appears from our data that the prevalent effector cells in this case are most likely lymphocytes and not monocytes, as evident from our results with enriched NK cells (Table 2). This contention is strengthened by the observation that nonadherent PBMC, such as those that failed to adhere to fibronectin, were more effective in lysing neuroblastoma cells coated with ch14.18 than adherent PBMC. Since fibronectin receptors are present on monocytes, but not lymphocytes (24), the more effective nonadherent cells are likely to be lymphocytes, rather than monocytes. This contention was confirmed by microfluorometric analysis, indicating that cells within the adherent population are ≥90% $CD14^+$, <1% $CD3^+$ cells, and <0.1% $CD57^+$, while the nonadherent lymphocytes consist of 65–80% $CD3^+$ and 5–10% $CD57^+$ cells with <1% $CD14^+$ monocytes.

However, the participation of monocytes in the ch14.18-mediated lysis of neuroblastoma cells cannot be completely ruled out, since only a 4-h lytic assay was used in our studies. In this regard, others have reported monocytes as effector cells in chimeric antibody-mediated lysis of colorectal carcinoma cells in lytic assays involving incubation times of at least 18 h (25). Even though examples can be found where monocytes serve as effector cells in ADCC of certain tumor cell lines, it appears that at least in our study of neuroblastoma, monocytes play a very minor role as effector cells in ADCC. This contention is supported in part by findings of Munn and Cheung (28), who were also unable to lyse neuroblastoma tumor cells with monocytes in the presence of murine anti-G_{D2} mAb 3F8, even in an 18-h lytic assay. Only after exposing monocytes to M-CSF for at least 4 days did these investigators observe monocytes to significantly lyse neuroblastoma cell lines in the presence of antibody. Thus, it is possible that cytotoxicity mediated by monocytes that is sufficient to effect lysis of neuroblastoma cells may depend on M-CSF or other cytokines for their activation and/or maturation to macrophages.

In further delineating other potent effector cells capable of lysing neuroblastoma cells coated with ch14.18, it became evident from the results of our study that granulocytes are more effective than PBMC in ADCC of neuroblastoma cells. An explanation is still lacking for the augmented ability of granulocytes to lyse neuroblastoma cells in the presence of ch14.18. One possible explanation for this finding may be that structural variations in the Fc γ receptor of granulocytes and PBMC could potentially influence their functional activities (29), making granulocytes better effector cells in ADCC. The possibility also exists that Fc γ receptor may be expressed at higher density on granulocytes than on PBMC or that granulocytes have a more efficient mechanism than PBMC for the lysis of neuroblastoma cells coated with ch14.18. One additional factor to be considered in interpreting the data presented in Fig. 5 is that the purification protocol used in this study could activate granulocytes. In this regard, other investigators have demonstrated that isolation of granulocytes on Ficoll gradients can enhance activation of these cells (30). Thus, even though the intrinsic properties of PBMC and granulocytes could explain the difference observed in their ability to lyse antibody-coated neuroblastoma cells, it is also possible that granulocytes could be activated during their purification.

Granulocytes, unlike PBMC, were able to lyse neuroblastoma cells equally well, whether the cells were coated with murine or chimeric anti-G_{D2} antibody, provided that the antibody concentration was sufficiently high, *i.e.*, ≥0.1 μg. One explanation for this finding could be that granulocyte-mediated lysis in the presence of ch14.18 has reached its plateau at the high antibody concentrations so that th murine anti-G_{D2} antibody appears to be equally capable of lysing neuroblastoma cells as its human/mouse chimeric counterpart. Thus, only at lower antibody concentrations is the human/mouse chimeric anti-G_{D2} antibody superior to the murine antibody.

The magnitude of ch14.18-mediated lysis of neuroblastoma was augmented further in the presence of GM-CSF. These findings are in agreement with the findings of other investigators who have shown that GM-CSF can enhance granulocyte-mediated lysis of neuroblastoma, which were coated with other anti-G_{D2} mAbs (31, 32). One mechanism by which GM-CSF can enhance ADCC of tumor cells is by increasing the production of toxic oxygen metabolites by granulocytes; however, granulocyte-mediated lysis of neuroblastoma in the presence of antibody is not dependent on these oxygen products for tumor cell lysis (32). Another possible explanation for the enhancing effect of GM-CSF on granulocyte-mediated cytotoxicity of mAb-coated neuroblastoma may be an increase in the number of Fc γ receptor on granulocytes, thus resulting in greater antibody binding.

Although most of our experiments involved the use of blood from normal adult individuals as a source of effector cells, we considered it important for two reasons to also evaluate effector cells obtained from neuroblastoma patients. First, it has been reported that effector cell functions are usually suppressed in neuroblastoma patients (33, 34). Second, many of the chemotherapeutic drugs used in the treatment of neuroblastoma induce immunosuppression, as well as neutropenia. In this study, we could demonstrate that PBMC from neuroblastoma patients are capable of lysing neuroblastoma cells with a greater efficiency when the tumor cells are coated with ch14.18 rather than with murine mAb 14.G2a. However, granulocytes from neuroblastoma patients can lyse neuroblastoma cells coated with ch14.18 far more effectively than PBMC obtained from the blood of these same patients. These findings indicate that effector cells of neuroblastoma patients can carry out similar tasks as those obtained from normal adults. Therefore, whatever factors may down-regulate other components of the immune system, they apparently do not influence the ability of neuroblastoma patients' effector cells to mediate lysis of tumor cells that are coated with ch14.18.

Since this study demonstrated that GM-CSF enhances ch14.18-mediated lysis of neuroblastoma cells by granulocytes from neuroblastoma patients, this augmentation of lysis could be important in increasing the ability of ch14.18 to ablate the growth of neuroblastoma. Although Massuci *et al.* (35) reported that GM-CSF can enhance mAb 17-1A-mediated lysis of human colorectal carcinoma cells by both lymphocytes and monocytes, GM-CSF was ineffective in our hands in enhancing the ability of PBMC to lyse ch14.18-coated neuroblastoma cells. The reason for the discrepancy between these two studies is not known.

Aside from enhancing antibody-mediated lysis of tumor cells by granulocytes, GM-CSF can also affect the migration of granulocytes (36). Therefore, a combined therapy of neuroblastoma patients with the human/mouse chimeric antibody

ch14.18 and GM-CSF could be beneficial, since GM-CSF may focus activated granulocytes to the tumor and enhance their ability to suppress the gr wth f neuroblastoma cells, thus helping to optimize the antitumor effect of ch14.18. In this regard, preliminary clinical studies demonstrated that granulocytes isolated from the blood of neuroblastoma patients that had been treated with murine anti-G_{D2} mAb 14.G2a did preferentially migrate to the tumor site following a brief ex vivo activation with GM-CSF. Considering these effects of GM-CSF, together with its known ability to increase the production of neutrophils in patients suffering from neutropenia as a consequence of chemotherapy (37), it may be of considerable future interest to test the effect of a combined therapy with GM-CSF and anti-G_{D2} mAb in neuroblastoma patients.

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commentary

Commentary

Challenges to the development of antigen-specific breast cancer vaccines

Matthew J Scanlan* and Dirk Jäger†

*Ludwig Institute for Cancer Research, New York, USA
†II Medizinische Klinik, Frankfurt, Germany

Correspondence: Mathew Scanlan, Ludwig Institute for Cancer Research, New York Branch of Human Cancer Immunology at Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10021. Tel: +1 212 639 8481; fax: +1 212 717 3100; e-mail: scanlanm@mskcc.org

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Abstract

Continued progress in the development of antigen-specific breast cancer vaccines depends on the identification of appropriate target antigens, the establishment of effective immunization strategies, and the ability to circumvent immune escape mechanisms. Methods such as T cell epitope cloning and serological expression cloning (SEREX) have led to the identification of a number target antigens expressed in breast cancer. Improved immunization strategies, such as using dendritic cells to present tumor-associated antigens to T lymphocytes, have been shown to induce antigen-specific T cell responses *in vivo* and, in some cases, objective clinical responses. An outcome of successful tumor immunity is the evolution of antigen-loss tumor variants. The development of a polyvalent breast cancer vaccine, directed against a panel of tumor-associated antigens, may counteract this form of immune escape.

Keywords: cancer vaccines, dendritic cells, immunotherapy, tumor antigens

Introduction

Convincing evidence for immune recognition of cancer in the autologous host has been provided by the identification of human tumor antigens [1,2] and by the verification of cancer immunesurveillance [3]. Cancer vaccines are a direct application of this knowledge and are based on the principle that a rigorous autotumorlytic immune response can be induced in cancer patients by immunization with tumor-associated antigens. Successful development of immunotherapeutic breast cancer vaccines hinges on the identification of appropriate target antigens and the establishment of effective immunization strategies, as well as on our ability to devise methods to circumvent immune escape mechanisms utilized by the evolving tumor. Preliminary progress in meeting these challenges is being made, as demonstrated by the ability of cancer vaccines to induce antigen-specific T lymphocyte responses and objective clinical responses in cancer patients. Although the results of recent clinical trials are promising, it should be noted that these are early-stage vaccine trials involving small populations of mostly end-stage melanoma patients, and are subject to variable patient and tumor responses. Nevertheless, the lessons learned from these studies can now be applied to the development of therapeutic breast cancer vaccines.

Target molecules for antigen-specific breast canc r vaccin s

Both immunogenicity in cancer patients and restricted tissue expression are characteristics used to define anti-

CT-7 = cancer testis-7; HLA = human leukocyte antigen; MHC = major histocompatibility complex; NY-ESO-1 = New York Esophageal-1; SSX = synovial sarcoma-x; TGF = transforming growth factor.

Table 1

Potential targets for antigen-specific breast cancer vaccines and their frequency of mRNA expression, amplification/overexpression[1] or mutation[2] in breast cancer

Target antigen	Expression in breast cancer	Reference
Differentiation antigens		
Carcinoembryonic antigen	50%	[5]
NY-BR-1	80%	[26]
Cancer–testis antigens		
NY-ESO-1	24%	[27]
MAGE-1	8%	[27]
MAGE-3	14%	[27]
BAGE	2%	[27]
GAGE	8%	[27]
SCP-1	31%	[27]
SSX-1	12%	[27]
SSX-2	8%	[27]
SSX-4	14%	[27]
CT-7	30%	[28]
Amplified/overexpressed antigens		
Her2/neu	40%[1]	[29]
NY-BR-62	60%[1]	[30]
NY-BR-85	90%[1]	[30]
Tumor protein D52	60%[1]	[30]
Mutational antigens		
p53	17%[2]	[31]

genic targets for cancer vaccines. Immunological methods of gene discovery, such as CD8+ and CD4+ T cell epitope cloning [1,4] and serum antibody expression cloning (SEREX) [2], have led to the identification of tissue-restricted tumor antigens that are recognized by the immune systems of cancer patients and have added to the list of target antigens applicable to breast cancer (Table 1). These antigens fall into several categories, such as differentiation antigens, cancer-testis antigens, amplified/overexpressed gene products, and mutational antigens. One of the first target molecules to be examined in the context of a breast cancer vaccine is carcinoembrionic antigen (CEA), a differentiation antigen of the gut, expressed exclusively in normal colonic epithelium and approximately 50% of breast cancers [5]. With regard to clinical trials, Morse and colleagues have observed objective responses in patients with metastatic disease, including breast cancer, following immunization with dendritic cells (see below) pulsed with an human leukocyte antigen (HLA)-A2 restricted peptide of CEA [6]. Recently, a new differentiation antigen of the breast, NY-BR-1, was identified by SEREX analysis and was found to be expressed exclusively in normal testis and breast, as well as in 80% of breast cancers (Jäger *et al*, manuscript submitted). NY-BR-1 is recognized by high titered serum IgG antibodies present in breast cancer patients, and its ability to induce a cellular immune response is under investigation.

Cancer-testis antigens represent a group of immunogenic proteins expressed exclusively in normal germ cells of the testis and embryonic ovary, and a percentage of various cancers. The melanoma antigens MAGE, BAGE, and GAGE are prototype cancer-testis antigens, first identified by cloning epitopes recognized by CD8+ T lymphocytes of melanoma patients [1,7]. SEREX analysis has also led to the identification of cancer testis antigens, including New York Esophageal-1 (NY-ESO-1), cancer testis-7 (CT-7), and the synovial sarcoma-x (SSX) family of antigens [8]. The enormous potential of CT antigens as vaccine targets is based on their restricted expression pattern and their high frequency of immunogenicity in cancer patients. Results of recent clinical trials using NY-ESO-1 [9] and MAGE-3 [10] as target antigens have been promising in terms of inducing antigen-specific T cells *in vivo* and, in some cases, concomitant disease regressions.

Mutated and amplified gene products represent another group of target antigens. The *Her-2/neu* oncogene is amplified in approximately 40% of breast cancers, and Her-2/neu-specific T cell responses have been observed in patients vaccinated with major histocompatibility (MHC) class II binding peptides derived from Her-2/neu [11]. The p53 tumor suppressor gene is frequently mutated in breast cancer and is associated with an autologous antibody response in breast cancer patients [12]. The large number of different p53 mutations makes targeting mutated p53 epitopes impractical. On the other hand, mutations increase the cellular half-life of p53, causing it to be overexpressed in cancer, indicating that immunization with wild type p53 may be an alternative. In fact, cytotoxic T lymphocyte (CTL) clones reactive against wild type p53 were generated from precursors present in the peripheral blood lymphocytes of healthy individuals, and were capable of lyzing several human tumor cell lines [13]. Three additional antigens recognized by the humoral immune system of breast cancer patients, NY-BR-62, NY-BR-85, and tumor protein D52, were found to be overexpressed in 60%, 90%, and 60% of breast cancers, respectively (Scanlan *et al*, manuscript submitted). Their significance in relation to breast cancer vaccines is being investigated.

Immunization strategies

Target antigens must first be presented as processed peptides bound to MHC class I and class II molecules. Recognition of these MHC-peptide complexes on the surface of antigen presenting cells (APCs) by antigen-specific T lymphocytes, together with additional co-stimulation, leads to the proliferation of antigen-specific CD8+ and CD4+ T cells capable of lytic and immunostimulatory functions. Many antigen-specific cancer vaccines have been prepared as MHC class I binding peptides and administered intradermally, along with adjuvant and cytokines, in order to enhance uptake by APCs and augment the immune response. Recently, a MAGE-3 peptide vaccine

yielded encouraging clinical results [14]. In this study, 7 out of 25 melanoma patients showed significant tumor regressions following vaccination, although there was no evidence of a strong CTL response against the MAGE-3 peptide in these responding patients.

Improvements in the delivery and presentation of target antigens are ongoing and include such strategies as continuous antigen administration by lymph node perfusion (Kundig, personal communication), direct targeting of APCs with recombinant *Listeria monocytogenes* that has been engineered to express tumor-associated antigens [15], and dendritic cell (DC) vaccines [16]. DCs are highly proficient APCs, expressing elevated levels of MHC class I and class II molecules, as well as important co-stimulatory molecules, and they also produce a variety of immunostimulatory cytokines [16]. DCs can be generated *in vitro* from precursors present in peripheral blood and subsequently used to present tumor antigens *in vivo*, when pulsed with antigenic peptide or transfected with DNA constructs encoding appropriate antigens. Several clinical trials employing DC vaccines have been carried out and the results have been promising [6,11,17]. With regard to epithelial cancers, Murphy and colleagues used DCs pulsed with HLA-A2 binding peptides derived from prostate-specific membrane antigen to treat patients with prostate cancer and observed significant clinical responses in 8 out of 33 vaccinated patients [18], and in a subset of these responders, cytokine secretion and CTL activity was detected against the immunizing peptide [19].

Current methods of adoptive immunotherapy rely on *in vitro* immunization, whereby tumor infiltrating lymphocytes are harvested from surgical specimens and propagated *in vitro* in the presence of interleukin-2 (IL-2) and appropriate antigen. The resultant CTL clones are then reintroduced into the autologous patient. Encouraging results have been obtained with this method as well. In one such study, CTLs specific for the melanocyte differentiation antigen gp100 were generated by cultivating tumor infiltrating lymphocytes in the presence of interleukin-2 and gp100. Upon infusion of these CTLs into autologous melanoma patients, significant tumor regressions were observed [20].

Other immunization strategies include the use of DNA vaccines, either in the form of viruses (adenovirus, vaccinia virus) or naked DNA, to deliver genes encoding tumor antigens [21]. Such vectors contain the coding sequence for a particular target antigen and may also contain sequences encoding targeting motifs for MHC class I and class II pathways, immunostimulatory cytokines, and co-stimulatory molecules. One major concern with using viral vectors is the presence of neutralizing antiviral antibodies in the recipient, resulting from a prior immunization (eg smallpox vaccine), which would negate vaccination.

Circumventing the tumor's immunological escap m chanisms

In response to immunesurveillance or effective immunotherapy, tumor cells may evolve mechanisms that allow them to escape immune recognition. Such immunoselection can cause an outgrowth of tumor cell populations that have lost expression of a given target antigen [22]. The use of polyvalent vaccines, specific for several tumor-associated antigens, or vaccination with antigens required by the tumor for maintenance of its malignant phenotype (eg telomerase), may circumvent this form of immune escape. Tumor cells also secrete immunosuppressive cytokines such as transforming growth factor (TGF)-β and IL-10, which can inhibit T lymphocyte effector function. Animal models have shown that it is possible to block the inhibitory activity of TGF-β by using an antibody against TGF-β in conjunction with IL-2 [23]. Similarly, blocking of inhibitory co-stimulation, such as the interaction between the CTLA-4 molecule on the surface of activated T cells and the B7 molecule on APCs, may augment the immune response [24]. An additional and quite significant form of immune escape is the ability of tumor cells to evolve mechanisms that impede antigen processing and presentation. Much attention is currently being focused on the proteosome, a key component of the antigen processing pathway, and it is hoped that these studies will generate a more thorough understanding of antigen presentation, enabling us to design strategies to thwart this mode of immune escape [25].

Conclusion

Although active immunotherapy directed against specific target molecules expressed in cancer offers promise for cancer treatment, a considerable amount of research needs to be carried out before it can be considered a viable therapeutic option. Identification of additional target antigens will increase the number of individuals that can be treated with cancer vaccines and allow for the development of polyvalent vaccines, which may offset antigen loss by tumors. Different immunization strategies are the subject of current immunotherapy trials, and by placing emphasis on the route of specific immunostimulation these trials may result in enhanced immune responses and clinical outcomes. Finally, it is important that patient monitoring schemes be enhanced and standardized since it will allow for improved measurement of patient responses and direct comparison of different clinical trials.

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R fer nc s

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Malignant Progenitors From Patients With Acute Myelogenous Leukemia Are Sensitive to a Diphtheria Toxin–Granulocyte-Macrophage Colony-Stimulating Factor Fusion Protein

By Donna E. Hogge, Cheryl L. Willman, Robert J. Kreitman, Marc Berger, Philip D. Hall, Kenneth J. Kopecky, Chris McLain, Edward P. Tagge, Connie J. Eaves, and Arthur E. Frankel

We have previously demonstrated that human granulocyte-macrophage colony-stimulating factor (GM-CSF) fused to a truncated diphtheria toxin (DT388-GMCSF) kills acute myelogenous leukemia (AML) cell lines bearing the GM-CSF receptor. We now report that exposure of malignant cells from 50 different patients with AML for 48 hours in culture to DT388-GMCSF reduces by a median of 1.6 logs (range, 0 to 3.7 logs) the number of leukemic cells capable of forming colonies in semisolid media (leukemic colony-forming cells [CFU-L]) with a median IC_{50} of 3×10^{-12} mol/L (range, 5 to $>4,000 \times 10^{-12}$ mol/L). Furthermore, the cell kill is dependent on the presence of high-affinity GM-CSF receptors on leukemic blasts, because CFU-L from 27 of 28 AML samples expressing ≥35 GM-CSF receptors per cell were inhibited by the toxin, whereas the colony growth from all 4 leukemic samples (2 AML, 1 acute lymphoblastic leukemia [ALL], and 1 prolymphocytic leukemia [PLL]) that had less than 35 receptors per cell was unaffected by the drug. Sensitivity of CFU-L to DT388-GMCSF was seen regardless of the clinical responsiveness of the patient's leukemia to standard chemotherapy agents. In contrast, clonogenic cells from normal bone marrow formed colonies at near control numbers after exposure to much higher toxin concentrations (4×10^{-9} mol/L) than those required to kill CFU-L from most patients. Thus, leukemic progenitors isolated directly from the peripheral blood of most AML patients show the same sensitivity to DT388-GMCSF as previously demonstrated for AML cell lines. Under the same conditions of exposure, normal hematopoietic progenitors are relatively unaffected by DT388-GMCSF, suggesting its potential as a therapeutic agent in AML.

ACUTE MYELOGENOUS leukemia (AML), the most common acute leukemia in adults, is associated with a 70% complete remission (CR) rate after standard induction chemotherapy regimens.[1] Intensive postremission therapy in combination with allogeneic bone marrow transplantation offers the possibility of cure to some of these individuals; however, fewer than 20% of all patients with the diagnosis of AML will have prolonged disease-free survival.[2] Resistance to standard chemotherapeutic drugs is an important cause of the relapsed, refractory leukemia to which most patients succumb.[3]

A number of identified drug resistance phenotypes are due to overexpression of specific proteins, and the concentration of these molecules in leukemic blasts has been correlated with response to cytotoxic chemotherapy.[4-8] In several cases, the resistance protein transports or inactivates xenobiotics such as anthracyclines. Pharmacologic reversal of MDR-1–related anthracycline resistance has, to date, been associated with toxicities to marrow, gastrointestinal tract, and the central nervous system; altered anthracycline pharmacodynamics; and minimal improvements in response rate or disease-free survival.[9] An antibody-targeted cytotoxic drug, anti-CD33-calicheamicin, was recently tested in a clinical trial and had reduced side effects.[10] However, most patients rapidly developed resistance due to active drug efflux. Thus, novel agents that are cytotoxic to leukemic blasts and bypass multidrug resistance phenotypes are urgently needed.

One such class of therapeutics are protein toxins covalently linked to peptide ligands. The ligand directs the molecule to the surface of specific cell types. The toxin moiety then enters the cell and catalytically inactivates protein synthesis. Toxins constructed that target AML blasts include the following: anti-CD33-blocked ricin, anti-CD33-gelonin, diphtheria toxin-interleukin-3 (IL-3), anti-transferrin receptor-ricin A chain, granulocyte-macrophage colony-stimulating factor (GM-CSF)-ricin, GM-CSF-Pseudomonas exotoxin, and diphtheria toxin-GM-CSF.[11-21] Each of these reagents inhibited protein synthesis by 50% (IC_{50}) in cell lines in a dose- and time-dependent manner. The least active drugs were anti-transferrin receptor-ricin A chain, GM-CSF-ricin, and GM-CSF-Pseudomonas exotoxin, with IC_{50}s of approximately 3×10^{-10} mol/L. Anti-CD33-blocked ricin and anti-CD33-gelonin had intermediate IC_{50}s of 10^{-10} mol/L, and diphtheria toxin-IL3 and diphtheria toxin-GM-CSF produced IC_{50}s of 3×10^{-11} mol/L. The variability in sensitivity that AML cell lines show to targeted toxins has been attributed to premature intracellular routing of ricin, ricin A chain, gelonin, and Pseudomonas exotoxin conjugates to lysomes. In contrast, the diphtheria toxin fusion proteins are able to translocate to the cytosol from a prelysosomal intracellular compartment. Specificity of cell kill has been demonstrated for several of these conjugates, including anti-CD33-blocked ricin, anti-CD33-gelonin, diphtheria toxin-IL3, and diphtheria toxin-GM-CSF. Reductions in normal human or murine marrow progenitors were seen after toxin conjugate exposure, but, in

From the Terry Fox Laboratory, British Columbia Cancer Agency, Vancouver, British Columbia, Canada; the Department of Pathology, University of New Mexico Cancer Center, Albuquerque, NM; the Laboratory of Molecular Biology, National Cancer Institute, National Institutes of Health, Bethesda, MD; the Departments of Surgery and Pharmaceutical Sciences, Medical University of South Carolina, Charleston, SC; the SWOG Statistical Center, Fred Hutchinson Cancer Research Center, Seattle, WA; and the Comprehensive Cancer Center at Wake Forest, Winston Salem, NC.

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Address reprint requests to Arthur E. Frankel, MD, Hanes 4046, Med Center Drive, Winston Salem, NC 27157.

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each case, the effect was much smaller than seen with AML cell lines.[17,19,20]

A critical question is whether these chimeric proteins, which require functional cell surface receptors, intact intracellular routing pathways, and sensitive protein synthesis machinery for intoxication, will kill malignant cells isolated directly from patients with leukemia. With several previously studied lymphoid malignancy-targeted toxins, including anti-CD5-ricin A chain and anti-CD25 (sFv)-Pseudomonas exotoxin, fresh leukemia cells were much less sensitive than cell lines.[22,23] Cytotoxicity was enhanced for anti-CD5-ricin A chain by adding the monocarboxylic acid ionophore, monensin, and for anti-CD25-Pseudomonas exotoxin by prolonged incubation. Both of these interventions improve intracellular toxin transport to compartments optimal for translocation to the cytosol. Few similar experiments have been conducted with toxins targeted to myeloid leukemia.

Roy et al[11] tested the cytotoxicity of anti-CD33-blocked ricin to leukemic colony-forming cells (CFU-L) from 12 AML patients and found that reductions in CFU-L colony formation were dose- and time-dependent. Perentesis et al[21] observed a 1 to 3 log kill of CFU-L from 7 of 9 therapy-refractory AML patients after exposure to diphtheria toxin-GM-CSF. However, it is possible that some of the observed effects in the latter studies were due to the presence of GM-CSF in the control but not in the toxin-treated test cultures.

To perform a comprehensive study of the sensitivity of leukemic clonogenic cells to diphtheria toxin-GM-CSF, we collected a series of 50 AML samples representing every French-American-British (FAB) subtype except M3 and M6. These included samples from patients who were known at the time to have disease both sensitive to and refractory to conventional cytotoxic agents. Also included were several samples from patients with lymphoid leukemias and normal bone marrow controls. We determined the GM-CSF receptor density and kd for the malignant blasts from each of the malignant samples and then assessed the ability of DT388-GMCSF to inhibit the growth of normal and leukemic clonogenic cells in standard semisolid growth factor-supplemented medium. As expected, some of the (untreated) leukemic cell samples failed to yield any discrete colonies in this assay. Nevertheless, for 33 of the 53 samples, we were able to determine for the toxin both the log kill and IC_{50} of CFU-L. The results demonstrate that leukemic progenitors from the majority of primary human AML patients are sensitive to the cytotoxic effects of this conjugate, regardless of their responsiveness to conventional chemotherapy drugs, whereas normal clonogenic cells largely are not.

MATERIALS AND METHODS

Cells. Heparinized peripheral blood samples from 50 patients with a diagnosis of AML and 3 patients with lymphoid leukemias and normal bone marrow samples from 4 individuals donating marrow for allogeneic transplantation were obtained after informed consent was obtained (MUSC protocol #7123, Terry Fox Laboratory protocol #C96-0429, and SWOG protocol #8600). Low-density (<1.077 g/mL) cells were isolated using a Ficoll-Hypaque gradient.

Freshly isolated or thawed, cryopreserved leukemic samples were suspended in RPMI 1640 medium (Irvine Scientific, Santa Ana, CA) with 15% fetal calf serum (FCS). After incubation for 1 hour at 37°C/5% CO_2 in 75-cm^2 tissue culture flasks (Costar Scientific Corp, Cambridge, MA), the nonadherent cells were collected and mixed with 0.1 mL anti-CD2 immunobeads (Dynal, Oslo, Norway). The bead-cell mixture was gently rocked at 4°C for 30 minutes, and then $CD2^+$ cells were depleted by magnetic separation. The procedure removed greater than 95% of $CD2^+$ T cells from initial preparations based on flow cytometry using anti-CD3-phycoerythrin binding (data not shown). Cells were then counted and aliquoted for the GM-CSF receptor and DT388-GMCSF sensitivity studies described below.

Fusion toxin. DT388-GMCSF was prepared and purified as previously described.[17] Material was stored as aliquots at 840 μg/mL in phosphate-buffered saline (PBS) plus 1% human serum albumin at −20°C until used. The material used in this study was found to kill the HL60 human AML cell line with an IC_{50} of 2×10^{-12} mol/L in a 48-hour ^{3}H-thymidine incorporation assay and to produce a maximum 3.5 log depletion of HL60 cells forming colonies in semisolid medium.[15]

GM-CSF receptor density. Aliquots of 1 to 6×10^6 cells in RPMI 1640 plus 2.5% bovine serum albumin and 20 mmol/L HEPES and 0.2% sodium azide were mixed with varying amounts of ^{125}I Bolton-Hunter-labeled human GM-CSF (80 to 120 μCi/μg; NEX249; DuPont, Boston, MA) with or without excess (1,500 ng) cold GM-CSF (Immunex, Seattle, WA) in a total volume of 150 μL in 1.5-mL Eppendorf tubes. Cells were incubated at 37°C for 30 minutes and then layered over a 200 μL oil phthalate mixture (1 part dioctylphthalate and 1.5 parts dibutylphthalate; Aldrich, Milwaukee, WI). After centrifugation at 12,000 rpm for 1 minute in a microfuge at room temperature, both pellets and supernatants were saved and counted in an LKB-Wallac 1260 Multi-gamma counter (Gaithersburg, MD) gated for ^{125}I with 50% counting efficiency. Background cpm was calculated by linear extrapolation from incubations with excess cold GM-CSF. Scatchard plots of specific bound/free versus specific bound cpm were made. Receptor number/cell was calculated using the following equation: the value for the x intercept ÷ (specific activity in μCi/μg × the cell number × [4.2 $\times 10^{-8}$]). kd was calculated as the x intercept times 2.7×10^{-13} divided by the y intercept times the specific activity. The Statistical software package (Statsoft, Tulsa, OK) was used for linear regression with separate analysis of the 6 lowest concentration ^{125}I-GM-CSF points and the 4 highest concentration ^{125}I-GM-CSF points. Nonlinear regression using the Radlig program (Biosoft) confirmed estimates from Scatchard plots for 4 samples. Receptor densities of 0 were recorded based on the absence of specific ^{125}I binding or negative values for kd.

Sensitivity of CFU-L and normal bone marrow clonogenic cells (colony-forming cells [CFC]) to DT388-GMCSF. Sensitivities of fresh leukemic blast progenitors and normal bone marrow CFC to DT388-GMCSF were tested in suspension culture. Aliquots of 10^6 AML blasts or light density marrow cells were placed in suspension culture with different concentrations of DT388-GMCSF (0 to 4×10^{-9} mol/L) in 24-well flat-bottomed Costar plates. AML cells were cultured in 1 mL of RPMI 1640 with 20% FCS and 50 ng/mL granulocyte colony-stimulating factor (G-CSF; Amgen, Thousand Oaks, CA) plus 1 of 10 different toxin concentrations over the indicated range. Normal marrow cells were cultured with 1 of 4 different DT388-GMCSF concentrations over the same range in 0.5 mL serum-free medium (StemCell Technologies, Vancouver, British Columbia, Canada) without added cytokines. Suspension cultures without the fusion toxin but the same in all other respects served as the untreated controls for determining the percentage of survival of clonogenic cells from both leukemic and normal samples.

CFU-L assays. After 48 hours of incubation at 37°C in 5% CO_2, 100 μL from each AML suspension culture containing different concentrations of DT388-GMCSF was mixed with 3 mL RPMI + 15% FCS plus 50 ng/mL G-CSF and GM-CSF and 0.3% agarose (SeaPlaque;

FMC Bioproducts, Rockland, ME) and poured into 35-mm gridded petri dishes (Nunc, Naperville, IL). In some experiments, 10% medium conditioned by the 5637 human bladder carcinoma cell line was added in the agarose colony assay instead of recombinant growth factors. After 10 minutes at 4°C to solidify the medium, dishes were placed in humidified chambers at 37°C/5% CO_2 for 14 to 21 days, after which colonies containing greater than 20 cells were counted. Both the concentrations of toxin reducing colony formation by 50% (IC_{50}) and the maximal log cell kill compared with controls were calculated as previously described.[15]

In one experiment, cells from 4 AML samples treated with DT388-GMCSF were assayed for colony formation in parallel in both the agarose-based assay described above and in methylcellulose medium (StemCell Technologies) with 30% FCS and 3 U/mL human erythropoietin (Epo; StemCell), 10 ng/mL GM-CSF (Sandoz International, Basel, Switzerland), 10 ng/mL IL-3 (Sandoz), 50 ng/mL Steel factor (SF; Terry Fox Laboratories, Vancouver, British Columbia, Canada), and 50 ng/mL flt-3 ligand (FL; Immunex) with equivalent results. All subsequent assays were performed using the agarose-based assay and form the basis of the experiments reported here.

Assays for normal bone marrow CFC. After 4 to 48 hours of incubation in suspension culture, cells to be assayed for burst-forming units-erythroid (BFU-E), colony-forming units-erythroid (CFU-E), CFU–granulocyte-macrophage (CFU-GM), and CFU-granulocyte/macrophage/erythroid/megakaryocyte (CFU-GEMM) were plated in methylcellulose-containing medium supplemented with 30% FCS and 3 U/mL Epo (StemCell) to which was added 50 ng/mL SF and 20 ng/mL each of human IL-6 (Terry Fox Laboratories), IL-3, GM-CSF, and G-CSF (Amgen). After 18 to 21 days at 37°C, erythroid, GM, and multilineage colonies were scored in situ. CFU-megakaryocyte (CFU-Mk) were detected as previously described in a serum-free assay modified to use a collagen base rather than agarose, to which was added 10 ng/mL IL-3, 10 ng/mL IL-6, and 50 ng/mL thrombopoietin (Zymogenetics Inc, Seattle, WA).[24] After 18 to 21 days of incubation, Mk-containing colonies were identified in situ using immunocytochemical staining with an anti-CD41 monoclonal antibody (provided by P. Lansdorp, Terry Fox Laboratories) followed by alkaline phosphatase/anti-alkaline phosphatase detection.[24]

RESULTS

Clinical history of AML patients. Fifty previously untreated AML patients were studied. The age, type of leukemia, and responsiveness of the patient to chemotherapy after collection of the sample are shown in Table 1. There were 2, 7, 13, 11, 11, and 1 patient with the subtypes M0, M1, M2, M4, M5, and M7, respectively, whereas for 5 patients the FAB type was not specified. No M3 or M6 subtypes were represented. A large proportion (78%) had presenting white blood cell counts in excess of 50×10^9/L. The median age was 48.5 years (range, 9 to 82 years). Three non-AML samples (patients no. 51 and 52 with acute lymphoblastic leukemia [ALL] and patient no. 53 with prolymphocytic leukemia [PLL]) were also tested. Of the 47 patients who received remission induction chemotherapy, 22 (47%) achieved CR.

GM-CSF receptor density. Because the GM-CSF receptor population includes both high-affinity α, β chain complexes and low-affinity receptors consisting of the α subunit only, we analyzed the presence of both types of receptor complex on leukemic blasts. An example of one of the Scatchard plots is shown in Fig 1. The results for all 53 leukemic patients are

Table 1. Clinical Characteristics of Leukemia Patients

Patient No.	Sex	Age	Disease	WBC ($\times 10^9$/L)	Chemoresponsiveness
1	F	64	AML-M4	130	Stable resistant disease
2	F	48	AML-M4	135	CR with SWOG8600
3	M	48	AML-M5A	46	ED
4	M	61	AML-M5	158	CR with araC/VP16/mitoxantrone
5	F	39	AML-M2	111	CR with SWOG8600
6	M	45	AML	39	CR with SWOG8600
7	F	46	AML-M5B	163	ED
8	F	69	AML-M5	100	CR with idarub./araC
9	M	50	AML	203	ED
10	M	44	AML-M5	46	ED
11	F	44	AML-M4	139	PR with SWOG8600
12	F	68	AML-M4	74	CR with araC/VP16/mitoxantrone
13	M	58	AML-M4	154	ED
14	M	47	AML-M1	43	CR with SWOG8600
15	F	37	AML-M2	74	NASS
16	M	19	AML-M2	64	CR with SWOG8600
17	M	18	AML-M4	87	Stable resistant disease
18	M	82	AML-M4	56	Received palliative hydrea
19	M	60	AML-M4	69	CR with SWOG8600
20	F	22	AML-M2	42	NASS
21	F	19	AML-M5B	79	CR with SWOG8600
22	F	61	AML-M2	21	Stable resistant disease
23	M	59	AML-M4	76	ED
24	M	61	AML-M0	170	Stable resistant disease
25	M	62	AML-M4E	63	CR with SWOG8600
26	M	36	AML-M2	165	Stable resistant disease
27	M	54	AML-M7	26	Stable resistant disease
28	M	36	AML-M5	119	CR with SWOG8600
29	M	74	AML-M5	118	Received palliative hydrea
30	F	51	AML	47	Stable resistant disease
31	M	63	AML-M1	121	Stable resistant disease
32	F	75	AML-M5	215	CR with araC/VP16/mitoxantrone
33	M	52	AML-M2	196	CR with SWOG8600
34	F	65	AML-M4	151	Stable resistant disease
35	M	9	AML-M5A	150	CR with VP16/mitoxantrone
36	F	55	AML-M1	129	Stable resistant disease
37	M	62	AML-M1	79	CR with SWOG8600
38	M	28	AML-M2	38	CR with SWOG8600
39	M	39	AML-M2	83	CR with SWOG8600
40	M	52	AML-M1	199	CR with araC/VP16/mitoxantrone
41	M	23	AML-M1	90	CR with idarub./araC
42	M	50	AML-M2	44	Stable resistant disease
43	M	24	AML-M2	68	Stable resistant disease
44	F	62	AML	295	ED
45	F	27	AML-M0	120	Stable resistant disease
46	M	19	AML-M5A	43	Stable resistant disease
47	F	49	AML(2°)	30	Stable resistant disease
48	M	44	AML-M2	211	CR with SWOG8600
49	M	40	AML-M1	212	ED
50	M	46	AML-M2	53	CR with SWOG8600
51	M	36	ALL	120	Stable resistant disease
52	M	36	ALL	501	CR to VCR/Pred/Dauno/Lasp
53	M	83	PLL	86	Palliative steroids, leukapheresis

SWOG8600 induction is daunorubicin + cytosine arabinoside (normal or high dose).[35]

Abbreviations: ED, early death; CR, complete remission; PR, partial remission; ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; PLl, prolymphocytic leukemia; NASS, not adequately assessed.

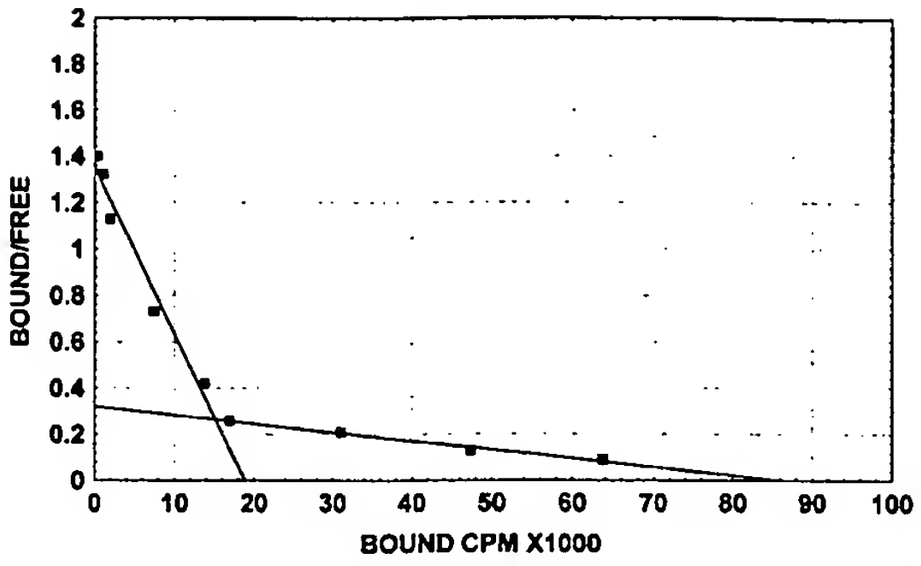

Fig 1. Scatchard plot of the blasts of patient no. 1 with 3.36 × 10^6 cells per aliquot and ^{125}I-GM-CSF specific activity of 84 μCi/μg. The lower 6 concentrations and the higher 4 concentrations were analyzed separately and the r^2 values on both are .98.

shown in Table 2. The PLL sample and 1 ALL sample (no. 52) had no detectable high-affinity receptors. The other ALL sample (no. 51) showed 93 high-affinity receptors/cell, with a kd of 4.5×10^{-11} mol/L and 216 low-affinity receptors, with a kd of 5×10^{-9} mol/L. Eighty-eight percent of AML patients (44/50) had ≥35 high-affinity receptors/cell, and 74% (37/50) had greater than 100 high-affinity receptors/cell. Among all 50 AML patients, for the high-affinity receptor, the mean ± SEM dissociation constant (kd) was $5 \pm 1.3 \times 10^{-11}$ mol/L, and the median kd was 2×10^{-11} mol/L. The mean and median kd of the low-affinity receptor were both $1 \pm 0.2 \times 10^{-9}$ mol/L. Nonlinear regression analysis yielded similar values (within 30%) for receptor numbers and kd on samples from 4 normal marrow donors.

Colony formation by fresh AML blasts. As shown in Table 2, 30 of 50 (60%) patient samples formed discrete colonies of greater than 20 cells after 14 to 21 days in semisolid medium. The plating efficiency of the various leukemic samples varied by almost 3 orders of magnitude (from 8 to 5,950 CFU-L per 10^5 cells plated).

Inhibition of blast colony formation by DT388-GMCSF. DT388-GMCSF reduced blast colony formation by at least 50% from 27 of 30 (90%, with 95% confidence interval 78%-98%) AML patient samples (Table 2 and Fig 2). It had no effect on colony formation from 2 AML samples (no. 46 and 47) that had less than 35 GM-CSF high-affinity receptors/cell. In contrast, the fusion toxin inhibited colony growth by at least 50% from all but 1 AML sample with ≥35 high-affinity receptors/cell (no. 25). The 3 non-AML patients' blasts were either insensitive (no. 53 and 52) or showed minimal sensitivity to the toxin (no. 51). The median IC_{50} for CFU-L among the 30 AML samples that formed colonies was 4×10^{-12} mol/L (range, 5 to >4,000 × 10^{-12} mol/L), whereas the corresponding values for log kill of CFU-L in the same samples was 1.6 (range, 0 to 3.7; Table 2). In logistic regression analyses based on the 25 AML patients who received remission induction chemotherapy, who had ≥ 35 high-affinity receptors per cell, and whose samples grew colonies, there were no significant associations between inhibition of colony growth (log cell kill) and probability of complete response (two-tailed $P = .36$) or of resistant disease ($P = .34$). Similarly, there was no significant association of the IC50 with CR ($P = .33$) or resistant disease ($P = .42$).

Table 2. GM-CSF Receptors and the Effect of DT388-GMCSF on Leukemic Cells

	High-Affinity Receptors		Low-Affinity Receptors		CFU-L		
Patient No.	No. per Cell	kd × 10^{-11}	No. per Cell	kd × 10^{-11}	No. per 10^5 Cells	IC_{50} × 10^{-13} mol/L	Maximum Log Kill
1	1,578	4.6	6,563	77	199	200	1
2	1,480	5	5,268	80	16	6	1.3
3	1,466	3	96,908	1,000	16	6	1.2
4	1,175	10	2,058	90	83	20	1.6
5	1,067	3	6,000	140	40	200	1.6
6	1,060	4	8,591	140	62	6	1.6
7	848	40	973	50	8	100	0.8
8	793	30	2,028	150	0	ND	ND
9	467	2.6	1,661	100	14	100	1
10	467	6	1,486	60	0	ND	ND
11	451	4.5	1,036	46	0	ND	ND
12	426	4.5	13,254	300	358	20	2.2
13	417	2	8,419	150	0	ND	ND
14	364	1.5	7,097	100	67	2,000	1.7
15	354	3.6	776	40	38	6	1.5
16	339	2	1,304	40	0	ND	ND
17	305	2.7	1,487	80	457	6	2.7
18	281	1	2,346	60	61	10	1.7
19	271	1	2,168	90	0	ND	ND
20	231	1.6	2,323	140	0	ND	ND
21	216	2	1,826	97	0	ND	ND
22	198	0.9	2,366	100	0	ND	ND
23	190	3	0	0	8	160	0.5
24	188	2	1,498	160	68	5	1.5
25	185	2	0	0	380	100	0.3
26	184	45	191	48	510	6	2
27	178	3	0	0	35	160	1.5
28	173	2	2,245	150	391	20	2.5
29	151	1.6	3,473	100	13	30	1.0
30	149	1	0	0	0	ND	ND
31	143	2	1,314	86	0	ND	ND
32	142	3.6	702	100	158	20	2.3
33	139	1	0	0	0	ND	ND
34	136	3	1,904	400	1,850	5	3.4
35	130	20	339	100	5,950	20	3.7
36	124	1.4	1,307	200	517	50	2.7
37	118	3	0	0	0	ND	ND
38	97	1	1,200	100	0	ND	ND
39	97	1.6	0	0	0	ND	ND
40	92	5	259	90	1,166	60	3.2
41	77	2	0	0	1,310	50	3.2
42	64	3	0	0	0	ND	ND
43	58	2	0	0	668	50	2.6
44	35	1	0	0	70	200	1.3
45	33	0.3	0	0	0	ND	ND
46	33	1	0	0	8	>40,000	0
47	6	ND	ND	ND	14	>40,000	0
48	0	0	0	0	0	ND	ND
49	0	0	0	0	0	ND	ND
50	0	0	0	0	0	ND	ND
51	93	4.5	216	49	13	6,000	1
52	0	0	0	0	33	>40,000	0
53	0	0	0	0	156	>40,000	0

Patient samples from MUSC, Terry Fox Lab, and SWOG processed as described in the text. Assays performed as described in text. The r^2 values for the high-affinity receptor plot ranged from .26 to .99, with a mean of .61, a median of .57, and a standard deviation of .17.

Abbreviation: ND, not determined.

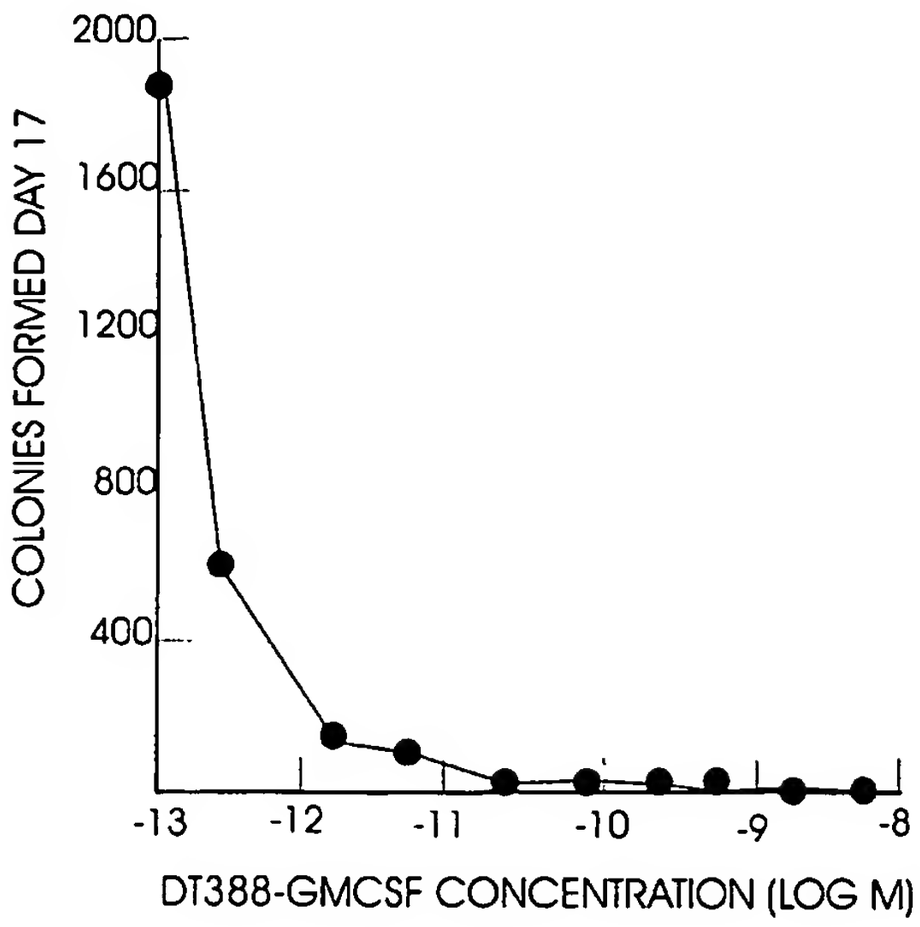

Fig 2. Colony growth inhibition after exposure of cells to DT388-GMCSF in liquid culture for 48 hours followed by their plating in semisolid medium at 17 days (patient no. 34).

Sensitivity of normal CFC to DT388-GMCSF. Light-density clonogenic cells from 4 normal bone marrow samples were also tested for sensitivity to the fusion toxin. As shown in Table 3, an average of 56% of total normal CFC survived (range, 33% to 102%), regardless of their lineage, after being incubated for 48 hours with the maximum concentration of DT388-GMCSF tested against both normal and malignant cells (4×10^{-9} mol/L). Failure of the fusion toxin to kill normal CFC was not attributable to competition from endogenous GM-CSF release during the 48-hour incubation period, because coincubated HL60 cells showed the same percentage of kill as in the absence of marrow cells (data not shown).

DISCUSSION

Recent clinical trials continue to show that the majority of AML patients develop disease resistant to standard cytotoxic chemotherapy drugs.[25,26] Because DT388-GMCSF works by a unique mechanism (inhibition of protein synthesis), its efficacy should not be affected by most multidrug resistance phenotypes,[15,17] and its in vivo toxicity profile may be different than current cytotoxic chemoradiotherapy regimens.[27] Thus, such a reagent could be useful in the treatment of both newly diagnosed and relapsed/refractory patients.

Table 3. Effect of DT388-GMCSF on Normal Bone Marrow CFC

Progenitor	n	% Surviving* (X ± SEM)
CFU-E	4	48 ± 31
BFU-E	4	64 ± 24
CFU-GM	4	66 ± 27
CFU-GEMM	4	40 ± 8
CFU-Mk	3	69 ± 87
Total CFC	4	56 ± 33

*After 48 hours of incubation with DT388-GMCSF at 4×10^{-9} mol/L by comparison to controls incubated under the same conditions but in the absence of DT388-GMCSF. The values for total CFC, CFU-E, BFU-E, CFU-GM, and CFU-Mk do not differ significantly from 100% ($P > .05$). The values for CFU-GEMM are significantly reduced from control values ($P < .001$).

Before further clinical development, we sought experimental evidence that AML blast progenitors from newly diagnosed patients could be killed by the fusion toxin. Although we intended to test toxin sensitivity in a series of different AML samples representative of the entire spectrum of phenotypes in this disease, the need for relatively large numbers of leukemic blasts to perform these experiments led to a selection bias for patients presenting with high circulating blasts counts. A large proportion of these patients (15/47 or 32%) failed to respond to standard remission induction chemotherapy, consistent with results from clinical trials showing that such patients have a poor prognosis.[25,26]

Almost all (47/50) of the AML patients in this study had GM-CSF receptors on their blasts. Our rate of receptor frequency paralleled those reported by others.[28,29] Kelleher et al[30] reported a slightly lower receptor frequency on a small group of patients using a cold saturation assay and two different types of recombinant GM-CSF. Our higher receptor frequency may reflect our use of the more sensitive hot saturation assay and/or the myelomonocytic differentiation of AML blasts from the M4 or M5 FAB subgroups, which made up half of the samples we tested.

Although it is well known that clonogenic progenitors exist among the malignant cells in patients with AML,[31] their frequency and the size and morphology of the colonies they produce are highly variable (Table 2).[32] To demonstrate the sensitivity of CFU-L to DT388-GMCSF quantitatively, it was necessary that the malignant blasts from a given sample form a sufficient number of discrete colonies in the semisolid assay used. Thirty of 50 (60%) AML samples analyzed here met this criterion. Conclusions regarding the sensitivity of CFU-L to the fusion toxin are thus restricted to this group. Similarly, the maximum log kill detected in these assays was, in many cases, determined by the clonogenicity of the untreated AML sample (ie, even if 100% of CFU-L were killed by the toxin at low concentrations, if only 10 colonies formed in the control assay the maximum log kill detectable is 1). Patients whose leukemic blasts contain a higher proportion of CFU-L have been reported to have a poorer prognosis than AML patients in general.[33] Nevertheless, even in this subgroup of patients, sensitivity of clonogenic progenitors to DT388-GMCSF was observed here, with at least 50% inhibition of colony growth in 90% of patients.

The presence of GM-CSF receptors on CFU-L against which the toxin showed cytotoxicity would be expected, because the drug must first bind to target cells and internalize before intoxication. Both subunits of the receptor must be present for ligand internalization.[34] In fact, AML blasts from all the samples against which DT388-GMCSF had activity were shown to have at least 35 high-affinity receptors per cell. We did not find a significant correlation between either the number of high-affinity receptors or their kd and their IC50 for the drug. With this study's limited sample size, there may have been insufficient statistical power to detect such associations. Another possible explanation for this fact is that the receptor

studies were performed on the entire blast population rather than on the small subset of clonogenic AML cells against which the toxicity of the drug was tested. These latter cells may differ from the population as a whole in their expression of GM-CSF receptors. Cells from the patient with PLL and 1 ALL patient were both receptor negative and insensitive to DT388-GMCSF, whereas the ALL patient sample that showed intermediate numbers of high-affinity receptors had modest sensitivity (IC_{50} 6×10^{-10} mol/L) to the drug. The blasts of the 2 AML patients (no. 46 and 47), with 6 and 33 high-affinity receptors/cell, were insensitive to DT388-GMCSF. Although these observations on lymphoid leukemias and AMLs with low numbers of GM-CSF receptors on circulating blasts should be confirmed with larger numbers, at present it appears appropriate to restrict future clinical development of this reagent to AML patients with GM-CSF receptor-positive blasts (≥35 receptors/cell).

CFU-L from the eight patients with refractory AML and blasts bearing ≥ 35 GM-CSF receptors per cell were assayed for DT388 GM-CSF sensitivity in this study. All eight showed significant (≥ 1 order of magnitude) inhibition of colony growth with low values of IC50 (eg, $< 2 \times 10^{-11}$ mol/L) for seven of the eight. These results extend an earlier report on nine refractory patients by Parentesis et al[21] in which seven had DT-GMCSF–sensitive blasts[21] and are consistent with our results on drug-resistant AML cell lines.[15,35] We hypothesize that targeted toxins are, in general, less affected by multidrug resistant phenotypes induced by conventional cytotoxic drugs or radiotherapy. Confirmation of this hypothesis will require testing of a larger group of relapsed/refractory patients' blasts.

In contrast to the marked toxicity of DT388-GMCSF on AML progenitors, its effect on most normal bone marrow clonogenic cells was insignificant, even after exposure to the highest concentrations of toxin tested for 48 hours. These results (Table 3) are consistent with those reported by others.[17,19,20] Further evidence for the lack of effect of this reagent on more primitive normal hematopoietic precursors that form cobblestone areas in long-term culture has also recently been published.[36] Taken together, these findings support the further preclinical development of DT388-GMCSF as a novel treatment agent for AML patients with both chemotherapy-sensitive and drug-resistant disease.

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(71) Applicant: MEDICAL UNIVERSITY OF SOUTH CAROLINA [US/US]; 171 Ashley Avenue, Charleston, SC 29425 (US).

(72) Inventor: FRANKEL, Arthur, Edward; 1509 Burning Tree Road, Charleston, SC 29412 (US).

(74) Agents: MILLER, Mary, L. et al.; Needle & Rosenberg, 127 Peachtree Street, N.E., Atlanta, GA 30303 (US).

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(57) Abstract

The present invention provides a plant holotoxin comprising 1α, 1β and 2γ subdomains and having a modification in a lectin binding site in each of the 1α, 1β and 2γ subdomains. In addition, the present invention provides a plant holotoxin fusion protein comprising a moiety consisting of a plant holotoxin comprising 1α, 1β and 2γ subdomains and having a modification in a lectin binding site in each of the 1α, 1β and 2γ subdomains and a moiety consisting of a ligand specific for a cell surface receptor. Furthermore, a method of constructing a ricin fusion protein immunotoxin is provided, comprising expressing the nucleic acid in a vector in a eukaryotic cell expression system to produce a fusion protein; isolating and purifying the fusion protein; and contacting the fusion protein of with a ricin toxin A chain under conditions which permit the association the fusion protein with the ricin toxin A chain. In addition, the present invention provides a method of treating a cancer or an autoimmune disease in a patient diagnosed with a cancer or an autoimmune disease comprising constructing a ricin fusion protein immunotoxin, wherein the ligand is specific for a particular cell surface receptor present only on the surfaces of the cancer cells or on the surfaces of the cells causing the patient's autoimmune disease; and administering the ricin fusion protein immunotoxin in a pharmaceutically acceptable carrier to the patient, whereby the ricin fusion protein immunotoxin treats the patient's cancer or autoimmune disease.

Ricin

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METHODS AND COMPOSITIONS FOR RICIN FUSION PROTEIN IMMUNOTOXINS TO TREAT CANCER AND AUTOIMMUNE DISEASE

BACKGROUND OF THE INVENTION

This invention was made with government support under the National Institutes of Health Grant number R01CA54116. The government has certain rights in the invention.

Field of the Invention

The present invention relates to a ricin fusion protein immunotoxin and methods for treating a cancer or autoimmune disease with this immunotoxin. In particular, the present invention provides a ricin fusion protein immunotoxin comprising a ricin toxin B chain from which three lectin binding sites have been removed, conjugated to a ligand specific for a particular cell surface receptor and associated with a ricin toxin A chain. Also provided are methods for administering the ricin fusion protein immunotoxin to treat a cancer or autoimmune disease in a patient diagnosed with a cancer or autoimmune disease.

Background Art

Ricin toxin, a glycoprotein produced in the seeds of *Ricinus communis* plants consists of a galactose-binding B chain (RTB) disulfide linked to an rRNA N-glycosidase A chain (RTA). The 65 kilodalton heterodimeric glycoprotein binds to cell surface galactose-terminated oligosaccharides via lectin binding sites in RTB (1) and undergoes receptor-mediated endocytosis (2). After trafficking to the Golgi (3), the toxin is transported to a distal compartment (4) from which the intersubunit disulfide bond is reduced (5). RTA then translocates to the cytosol and catalytically inactivates protein

synthesis by hydrolysis of a specific adenine base from the 26S ribosomal RNA (6). Galactose binding is important for cell binding and may be needed for internalization and intracellular trafficking of ricin (34).

Ricin toxin is one of the most toxic substances known to man. A single molecule is capable of causing cell death (7), and the LD_{50} of ricin in 20 gram C57/Bl6 mice is reported to be 60 nanograms or 6×10^{11} molecules (8). Histopathological examination of mice given toxic doses of ricin failed to show any definite abnormalities (8). Thus, the critical target organ for ricin is unknown.

The x-ray crystallographic structure of ricin revealed two domains each with three subdomains each with similar folding and primary amino acid sequence (13). While all six subdomains had α-carbon chains forming a loop, twist and hook, only four of the subdomains (1α, 1β, 2α, and 2γ) contained tripeptide kinks. Co-crystallization of 5 mM α-lactose with ricin showed sugar binding in the tripeptide kinks of subdomains 1α and 2γ.

Many patients with hematopoietic malignancies have incomplete responses to chemoradiotherapy and die from progressive disease. Patients' leukemic blasts may develop multiple drug resistance phenotypes and normal tissue toxicities may limit dose escalation.

Fusion toxins have been developed in an effort to treat these diseases. Fusion toxins are hybrid proteins composed of peptide ligands reactive with malignant cells (antibody fragments or cytokines) fused to polypeptide toxins [diphtheria toxin (DT), *Pseudomonas* exotoxin (PE) or ricin]. The toxin-ligand-receptor complex internalizes into intracellular compartments from which the catalytic domain of the toxin translocates to the cytosol and

inactivates protein synthesis. There have been several targets [including interleukin-2 receptors (IL2R), etc.] for which fusion toxins have been designed.

Although ricin fusion toxins have been made, the construction of these toxins has been hampered by the requirement for a reducible disulfide between RTA and the ligand for cell intoxication (30). Initial efforts to produce IL2-RTA fusions yielded nontoxic molecules. Subsequent efforts to introduce a diphtheria toxin loop peptide or factor Xa recognition sequence between IL2 and RTA did not yield disulfide linked molecules and were noncytotoxic to IL2R bearing cells (31). Ricin's extreme potency has led to its use in immunotoxins consisting of monoclonal antibodies chemically coupled to a modified ricin moiety. However, in published clinical trials, the large 200 kilodalton Mr immunoconjugates showed significant vascular endothelial toxicity (32). Three groups of investigators have chemically or genetically modified lectin sites on ricin and used covalently attached ligands to study cell intoxication (33-35). In each case, reductions in lectin function led to profound decreases in cytotoxic potency. However, despite these efforts, novel therapeutic modalities with minimal toxicities and no cross-resistance with current cytotoxic treatments are still needed.

Several other strategies have been used to target the IL2R in patients with leukemias and lymphomas., including antibodies to the α subunit of the interleukin-2 receptor (anti-IL2Rα) conjugated to PE, IL-2 fused to DT and anti-IL2Rα conjugated to RTA. Each of these strategies has yielded only partial success and resulted in some toxic side effects. For example, when RTA was conjugated to anti-IL2Rα and given intravenously to 14 Hodgkin's disease patients, only one partial remission was seen (39). Furthermore, vascular leak syndrome (VLS) with edema, weight gain, hypoalbuminemia and dyspnea was

dose-limiting. In a parallel approach, when IL2 was fused to fragments of DT (18,19) and given systemically to lymphoid malignancy patients, durable complete remissions were rare, suggesting the need for more potent and selective IL2R-directed therapies.

The present invention overcomes previous shortcomings by providing a ricin fusion protein immunotoxin comprising a ricin toxin B chain fusion protein having a modification in a lectin binding site in each of the 1α, 1β and 2γ subunits and a ligand specific for a cell surface receptor in association with a ricin toxin A chain that selectively targets and intoxicates very specific cell populations for the treatment of cancer and autoimmune disease.

SUMMARY OF THE INVENTION

The present invention provides a plant holotoxin comprising 1α, 1β and 2γ subdomains and having a modification in a lectin binding site in each of the 1α, 1β and 2γ subdomains, For example, a ricin toxin B chain having a modification in a lectin binding site in each of the 1α, 1β and 2γ subdomains is provided.

In addition, the present invention provides a plant holotoxin fusion protein comprising a moiety consisting of a plant holotoxin comprising 1α, 1β and 2γ subdomains and having a modification in a lectin binding site in each of the 1α, 1β and 2γ subdomains and a moiety consisting of a ligand specific for a cell surface receptor. For example, a ricin toxin B chain fusion protein comprising a moiety consisting of a ricin toxin B chain having a modification in a lectin binding site in each of the 1α, 1β and 2γ subdomains and a moiety consisting of a ligand specific for a cell surface receptor is provided.

Furthermore, a method of constructing a ricin fusion protein immunotoxin is provided, comprising expressing the nucleic acid in a vector in a eukaryotic cell expression system to produce a fusion protein; isolating and purifying the fusion protein; and contacting the fusion protein of with a ricin toxin A chain under conditions which permit the association of the fusion protein with the ricin toxin A chain.

In addition, the present invention provides a method of treating a cancer or an autoimmune disease in a patient diagnosed with a cancer or an autoimmune disease comprising constructing a ricin fusion protein immunotoxin, wherein the ligand is specific for a particular cell surface receptor present only on the surfaces of the cancer cells or on the surfaces of the cells causing the patient's autoimmune disease; and administering the ricin fusion protein immunotoxin in a pharmaceutically acceptable carrier to the patient, whereby the ricin fusion protein immunotoxin treats the patient's cancer or autoimmune disease.

Finally provided is a ricin protein immunotoxin comprising a ricin toxin A chain associated with a ricin toxin B chain fusion protein comprising a ricin toxin B chain having a W to S substitution at amino acid position 37 in the 1α subdomain, a Y to H substitution at amino acid position 248 in the 1β subdomain and a Y to H substitution at position 78 in the 2γ subdomain and a ligand specific for a cell surface receptor.

Various other objectives and advantages of the present invention will become apparent from the following description.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention may be understood more readily by reference to the following detailed description of specific embodiments and the Examples included herein. As used in the claims, "a" can include multiples.

The target for several leukemia-directed fusion toxins has been the interleukin-2 receptor (IL2R). IL2R is a heterotrimeric glycoprotein complex on the cell membrane with a 55 kD α subunit, a 75 kD β subunit and a 64 kD γ subunit (16). The only normal human tissues expressing IL2Rα and IL2Rβ are activated T cells, B cells, LGL cells and monocytes and some liver Kupffer cells, lung macrophages and skin Langerhans' cells. Thus, an immunotoxin targeted to this receptor is expected to be reasonably selective. A variety of hematologic neoplasms may show high affinity IL2R expression including hairy cell leukemia, adult T cell leukemia and a fraction of cutaneous T cell lymphomas and B-cell chronic lymphocytic leukemias (17). DT and PE have been fused to either IL2 or antibody Fv anti-IL2R peptides (18-25). All reagents showed potent selective cytotoxicities *in vitro* and in some cases, *in vivo*.

Ricin-based fusion proteins are attractive candidates for development for several reasons. First, the toxin inactivates cell protein synthesis by a mechanism independent of that used by DT or PE. The RNA N-glycosidase activity of ricin cripples 1500 ribosomes/minute and a single molecule of ricin in the cytosol can cause cell death (6,27). Thus, ricin fusion toxins may be used in combination with bacterial fusion toxins or when bacterial fusion toxin resistance is encountered. Furthermore, there is no immunologic cross-reactivity between ricin and the bacterial toxins. Patients who have been immunized with DT or had previous exposure to PE do not show amnestic

immune responses to ricin (28). Finally, there is extensive clinical experience with RTA and blocked ricin immunotoxins suggesting safety in patients (29).

The present invention provides a plant holotoxin comprising 1α, 1β and 2γ subdomains and having a modification in a lectin binding site in each of the 1α, 1β and 2γ subdomains. The plant holotoxin can be, but is not limited to, ricin, mistletoe toxin, abrin, volkensin as well as any other compound now known or identified in the future to be a plant holotoxin. The preferred plant holotoxin will be characterized as a 65 kD glycoprotein with a galactose lectin B chain and RNA N-glycosidase A chain. The plant holotoxin with the modification described herein can have various functional attributes, such as, for example, the ability to be produced in high yields as an expressed protein in a eukaryotic gene expression system; specific reactivity with antibodies against the plant holotoxin; proper folding to retain the stability and functional characteristics of the wild type holotoxin; proper association with additional moieties which are normally associated with the wild type holotoxin; a 50% lethal dose (LD_{50}) value of greater than ten micrograms in mice; at least a one thousand fold reduction in sugar binding as compared with wild type, at least a one hundred fold reduction in toxicity in mice as compared with wild type; and the ability to selectively intoxicate a target cell with a twenty fold to a greater than two hundred fold reduction in the ability to intoxicate a non-target cell, as compared with wild type. These functional attributes can be determined according to the protocols provided herein in the Examples.

The modification in a lectin binding site in each of the 1α, 1β and 2γ subdomains of the plant holotoxin can be an amino acid substitution, such as, for example, a substitution of an amino acid having an aromatic ring residue with an amino acid lacking an aromatic ring residue. Other modifications in the lectin binding sites can include, but are not limited to, for example, substitution

of an amino acid with a polar hydrogen binding residue with an amino acid with a nonpolar residue, as well as other deletions, additions or amino acid substitutions or any other modifications now known or later discovered that result in either complete or significant removal of the sugar binding activity of the lectin binding site. In the preferred embodiment, the plant holotoxin with modifications will maintain a functional conformation and be able to associate normally with the intoxication-imparting moiety. Whether a given modification results in the complete or significant removal of the sugar binding activity of a lectin binding site can be determined according to the protocols provided in the Examples herein.

Also provided in the present invention is a ricin toxin B chain having a modification in a lectin binding site in each of the 1α, 1β and 2γ subdomains. The ricin toxin B chain with such modification can have various functional attributes, such as, for example, the ability to be produced in high yields as an expressed protein in a eukaryotic gene expression system; specific reactivity with antibodies against the ricin toxin B chain; proper folding to retain the stability and functional characteristics of the wild type ricin toxin B chain; proper association with ricin toxin A chain; a 50% lethal dose (LD_{50}) value of greater than ten micrograms in mice; at least a one thousand fold reduction in sugar binding as compared with wild type ricin toxin B chain, at least a one hundred fold reduction in toxicity in mice as compared with wild type ricin toxin B chain; and the ability to selectively intoxicate a target cell with a two hundred fold reduction in the ability to intoxicate a non-target cell, as compared with wild type ricin toxin B chain. These functional attributes can be determined according to the protocols provided herein in the Examples.

The modification in a lectin binding site in each of the 1α, 1β and 2γ subdomains in the ricin toxin B chain of this invention can be an amino acid

substitution, such as, for example, wherein the amino acid substitution consists of substitution of an amino acid having an aromatic ring residue with an amino acid lacking an aromatic ring residue. Other modifications in the lectin binding sites can include, but are not limited to, for example, substitution of an amino acid with a polar hydrogen binding residue with an amino acid with a nonpolar residue, as well as other deletions, additions or amino acid substitutions or any other modifications now known or later discovered that result in either complete or significant removal of the sugar binding activity of the lectin binding site. Whether a given modification results in the complete or significant removal of the sugar binding activity of a lectin binding site can be determined according to the protocols provided in the Examples herein.

For example, in the ricin toxin B chain, the amino acid substitutions can consist of a W to S substitution at amino acid position 37 in the 1α subdomain, a Y to H substitution at amino acid position 248 in the 1β subdomain and a Y to H substitution at position 78 in the 2γ subdomain. Other modifications in the lectin binding sites can include, but are not limited to, for example, substitution of an amino acid with a polar hydrogen binding residue with an amino acid with a nonpolar residue, as well as other deletions, additions or amino acid substitutions or any other modifications now known or later discovered that result in either complete or significant removal of the sugar binding activity of the lectin binding site. Whether a given modification results in the complete or significant removal of the sugar binding activity of a lectin binding site can be determined according to the protocols provided in the Examples herein.

A plant holotoxin comprising 1α, 1β and 2γ subdomains and having a modification in a lectin binding site in one or more or the subdomains, including at least a modification in the 1β subdomain is also contemplated in the present

invention. The modification in the lectin binding site in one or more of the subdomains can be an amino acid substitution, such as for example, a substitution of an amino acid having an aromatic ring residue with an amino acid lacking an aromatic ring residue (e.g.,a Y to H substitution at amino acid position 248 in the 1β subdomain).

Further provided is a ricin toxin B chain fusion protein having a modification in a lectin binding site in one or more subdomain, including at least a modification in the 1β subdomain. The modification in one or more of the subdomains can be an amino acid substitution, such as, for example, a substitution of an amino acid having an aromatic ring residue with an amino acid lacking an aromatic ring residue. For example, the amino acid substitution can be, but is not limited to, a Y to H substitution at position 248 in the 1β subdomain.

Furthermore, a plant holotoxin fusion protein is provided, comprising a plant holotoxin comprising 1α, 1β and 2γ subdomains and having a modification in a lectin binding site in each of the 1α, 1β and 2γ subdomains and a ligand specific for a cell surface receptor. The ligand of the plant holotoxin fusion protein can be, but is not limited to, interleukin-2, granulocyte/macrophage colony stimulating factor, an antibody or antibody fragment to CD3, an antibody or antibody fragment to GD2, epidermal growth factor, IGF2, GRF, substance P, MSH, as well as any other molecular entity now known or identified in the future to be a ligand specific for a cell surface receptor as determined by assaying a potential ligand for selective binding avidity for a particular cell surface receptor by protocols standard in the art for measuring binding avidities.

The present invention additionally provides a ricin toxin B chain fusion protein comprising a ricin toxin B chain having a modification in a lectin binding site in each of the 1α, 1β and 2γ subdomains as described above and a ligand specific for a cell surface receptor. The ligand of the ricin toxin B chain fusion protein can be, but is not limited to, interleukin-2, granulocyte/macrophage colony stimulating factor, an antibody to CD3, an antibody to GD2, epidermal growth factor, an antibody or antibody fragment to CD3, an antibody or antibody fragment to GD2, epidermal growth factor, IGF2, GRF, substance P, MSH, as well as any other molecular entity now known or identified in the future to be a ligand specific for a cell surface receptor as determined by assaying a potential ligand for selective binding avidity for a particular cell surface receptor by protocols standard in the art for measuring binding avidities.

The present invention also provides a ricin toxin B chain fusion protein comprising a ricin toxin B chain having a W to S substitution at amino acid position 37 in the 1α subdomain, a Y to H substitution at amino acid position 248 in the 1β subdomain and a Y to H substitution at position 78 in the 2γ subdomain and a ligand specific for a cell surface receptor. Further provided is a ricin fusion protein immunotoxin comprising this ricin toxin B chain fusion protein associated with a ricin toxin A chain.

A plant holotoxin fusion protein immunotoxin is also provided in the present invention, comprising a plant holotoxin fusion protein (which imparts a binding function to the immunotoxin) as described above, associated with a moiety imparting an intoxicating function to the immunotoxin. For example, a ricin fusion protein immunotoxin is provided in the present invention, comprising a ricin toxin B chain fusion protein consisting of a ricin toxin B chain having a modification in a lectin binding site in each of the 1α, 1β and 2γ

subdomains and a ligand specific for a cell surface receptor, associated with a ricin toxin A chain.

Nucleic acids encoding the plant holotoxins and fusion proteins of this invention are also contemplated, as well as vectors comprising the nucleic acids and hosts comprising the vectors. The present invention also provides nucleic acids complementary to or capable of hybridizing with the nucleic acids encoding the plant holotoxins and fusion proteins.

The nucleic acid of the plant holotoxin or fusion protein can encode the intact plant holotoxin with the modifications described herein or an active fragment thereof. An active fragment of a plant holotoxin is a fragment which is capable of maintaining a functional conformation and associating with the moiety which imparts an intoxicating function to the plant holotoxin. The nucleic acid of the fusion protein can also encode the intact ligand or an active fragment thereof. An active fragment of a ligand of this invention is a fragment which is capable of maintaining a functional conformation and specifically binding to its corresponding cell surface receptor.

Protocols for construction of a vector containing a nucleic acid encoding a plant holotoxin fusion protein such as the ricin toxin B chain fusion protein are well known in the art and are described in the Examples provided herein. The vector can be expressed in any *in vitro* eukaryotic cell expression system, such as, for example, the *Spodoptera frugiperda* insect cell line which expresses proteins in a baculovirus vector, as described in the Examples herein. Isolation and purification of the expressed fusion protein can be carried out by protocols well known to those of skill in the art, e.g., as described in the Examples herein.

Also provided in the present invention is a method of constructing a ricin fusion protein immunotoxin comprising expressing a nucleic acid encoding the ricin toxin B fusion protein, in a vector in a eukaryotic cell expression system, to produce a ricin toxin B chain fusion protein; isolating and purifying the ricin toxin B chain fusion protein; and contacting the ricin toxin B chain fusion protein with a ricin toxin A chain under conditions which permit the association of the ricin toxin B chain fusion protein with the ricin toxin A chain.

The present invention further contemplates a method of constructing a plant holotoxin fusion protein immunotoxin comprising expressing a nucleic acid encoding the plant holotoxin fusion protein described above, in a vector in a eukaryotic cell expression system to produce a plant holotoxin fusion protein; isolating and purifying the plant holotoxin fusion protein; and contacting the plant holotoxin fusion protein with a moiety which imparts an intoxicating function, under conditions which permit the association of the plant holotoxin fusion protein with the intoxicating moiety to yield a plant holotoxin fusion protein immunotoxin.

In addition to the vectors and expression systems described in the Examples herein, a variety of vectors and eukaryotic expression systems such as yeast, filamentous fungi, insect cell lines, bird, fish, transgenic plants and mammalian cells, among others, as are known to those of ordinary skill in the art, can also be used in the present invention.

The vectors of the invention can be in a host (e.g., cell line or transgenic animal) that can express the nucleic acid contemplated by the present invention. As indicated above, the vector, *e.g.*, a plasmid, which is used to transform the host cell, preferably contains DNA sequences to initiate transcription and sequences to control the translation of the protein. These

sequences are referred to as expression control sequences. Suitable vectors for expression systems usually have expression control sequences, such as promoters, including 3-phosphoglycerate kinase or other glycolytic enzymes, an origin of replication, termination sequences and the like, as desired.

Preferred expression control sequences are promoters derived from immunoglobulin genes, SV40 virus, adenovirus, bovine papilloma virus, etc, as are well known in the art. For example, gene sequences to control replication in the host cell may be incorporated into the vector such as those found in bovine papilloma virus-type vectors (64). A variety of suitable vectors are described in the literature (see, for example, 65,66).

Appropriate vectors for expressing proteins in insect cells are usually derived from baculovirus. Suitable insect cell lines include, but are not limited to, mosquito larvae, silkworm, armyworm, moth and *Drosophila* cell lines such as a Schneider cell line (67), as well as any other insect cell line now known or identified in the future to be a suitable host cell line for baculovirus or other insect cell expression vectors.

When yeast or higher animal host cells are employed, polyadenylation or transcription terminator sequences from known mammalian genes can be incorporated into the vector. An example of a terminator sequence is the polyadenylation sequence from the bovine growth hormone gene. Sequences for accurate splicing of the transcript may also be included. An example of a splicing sequence is the VP1 intron from SV40 (68).

The nucleic acid sequences can be expressed in hosts after the sequences have been operably linked to, i.e., positioned, to ensure the functioning of an expression control sequence. These expression vectors are typically replicable in the host organisms either as episomes or as an integral

part of the host chromosomal DNA. Commonly, expression vectors can contain selection markers, e.g., tetracycline resistance, hygromycin resistance, gentamicin resistance or methotrexate resistance, to permit detection and/or selection of those cells transformed with the desired nucleic acid sequences (*see*, e.g., U.S. Patent 4,704,362). The presence of the vector RNA in transformed cells can be confirmed by Northern blot analysis and production of a cDNA or opposite strand RNA corresponding to the antigen coding sequence can be confirmed by Southern and Northern blot analysis, respectively.

Polynucleotides encoding a variant polypeptide may include sequences that facilitate transcription (expression sequences) and translation of the coding sequences such that the encoded polypeptide product is produced. Construction of such polynucleotides is well known in the art. For example, such polynucleotides can include a promoter, a transcription termination site (polyadenylation site in eukaryotic expression hosts), a ribosome binding site and optionally, an enhancer for use in eukaryotic expression hosts as well as any sequences necessary for replication of a vector.

The host cells are rendered competent for transformation by various means known in the art. There are several well-known methods of introducing DNA into eukaryotic cells. These include, but are not limited to, calcium phosphate precipitation, fusion of the recipient cells with bacterial protoplasts containing the DNA, treatment of the recipient cells with liposomes containing the DNA, DEAE dextran, electroporation, micro-injection of the DNA directly into the cells, as well as any other technique now known or developed in the future for introducing nucleic acid into cells.

The transformed cells are cultured by means well known to one of ordinary skill in the art (69). The expressed polypeptides are isolated from

cells grown as suspensions or monolayers. The latter are recovered by well-known mechanical, chemical, or enzymatic means and purified according to standard methods well known in the art.

Synthesis of heterologous proteins in yeast is well known. For example, Sherman *et al.* (70), is a well-recognized work describing the various methods available to produce a protein in yeast.

Two procedures are used in transforming yeast cells. In one case, yeast cells are first converted into protoplasts using zymolase, lytiacase, or glusulase, followed by addition of DNA and polyethylene glycol (PEG). The PEG-treated protoplasts are then regenerated in a 3% agar medium under selective conditions. Details of this procedure are described by Beggs, J.D. (71) and Hinnen *et al.* (72). The second procedure does not involve removal of the cell wall. Instead, the cells are treated with lithium chloride or acetate and PEG and put on selective plates (73).

Plant holotoxins and fusion proteins, once expressed, can be isolated from yeast by lysing the cells and applying standard protein isolation and purification techniques to the lysates. The monitoring of the purification process can be accomplished by using Western blot techniques or radioimmunoassay or other standard immunoassay techniques.

The sequences encoding plant holotoxins and fusion proteins of the present invention can also be ligated to various expression vectors for use in transforming cell cultures of, for example, mammalian, insect, plant, bird or fish origin. Illustrative of cell cultures useful for the production of polypeptides are mammalian cells. Mammalian cells permit the expression of proteins in an environment that favors important post-translational modifications such as

folding and cysteine pairing, addition of complex carbohydrate structures and secretion of active protein.

Mammalian cell systems can be in the form of monolayers of cells, although mammalian cell suspensions may also be used. A number of suitable host cell lines capable of expressing intact proteins have been developed in the art, and include the HEK293, BHK21 and CHO cell lines, as well as various human cells such as COS cell lines, HeLa cells, myeloma cell lines, Jurkat cells, etc., as are known in the art. Other animal cells useful for the production of proteins are available, for example, from the American Type Culture Collection Catalogue of Cell Lines and Hybridomas (7th Edition, 1992). Expression vectors for these cells can include expression control sequences, such as an origin of replication, a promoter (*e.g.*, the CMV promoter, a HSC tk promoter or pgk [phosphoglycerate kinase] promoter), an enhancer (74) and necessary processing information sites, such as ribosome binding sites, RNA splice sites, polyadenylation sites (*e.g.*, an SV40 large T antigen poly A addition site) and transcriptional terminator sequences.

Efficient post translational glycosylation and expression of recombinant proteins can also be achieved in insect cell expression systems employing baculovirus vectors, as described in the Examples herein.

The plant holotoxin of this invention can also be expressed in transgenic plant expression systems known in the art, such as, for example, soy bean cells or *Nicotiana tabacum* cells (83).

The nucleic acids of the present invention can be used to generate transgenic nonhuman animals in which the nucleic acid encoding a plant holotoxin or fusion protein of the present invention is added to the germ line of

the animal. Thus a cell of the invention containing an nucleic acid of this invention is contemplated to include a cell in a transgenic animal. The plant holotoxin or fusion protein can be isolated and purified from materials secreted by the animal, such as for example, milk secreted from nonhuman mammals. Transgenic animals are generated by standard means known to those skilled in the art (see, for example, 84)

Ricin toxin A chain can be obtained commercially or by expression of a nucleic acid encoding ricin toxin A chain in prokaryotic or eukaryotic *in vitro* expression systems according to standard protocols known in the art and associated with the ricin toxin B chain fusion protein to produce the immunotoxin (82). The nucleic acid encoding the ricin toxin A chain can also be included in a vector which also comprises the nucleic acid encoding the ricin toxin B chain fusion protein of this invention. This vector can be introduced into a eukaryotic expression system, such as a plant cell expression system (83) under conditions whereby the ricin toxin A chain and the ricin toxin B chain fusion protein are produced and associate in the same cell, allowing for isolation and purification of the complete immunotoxin from a single expression system.

As used herein to describe the interactions between ligands and cell surface receptors, interactions between antibodies and antigens or interactions between immunotoxins and target cells, the terms "selective" or "selectively" and "specific" or "specifically" all have the same meaning and are thus used interchangeably to mean either that the ligand binds to only one type of cell surface receptor which has been identified as binding that particular ligand and does not randomly bind to other cell surface molecules, that the antibody binds its corresponding antigen which has been identified and does not randomly bind

with other antigens, or that the immunotoxin binds its intended target cell which has been identified and does not randomly bind to other nontarget cells.

Also contemplated for the present invention is a method for treating or preventing a cancer or an autoimmune disease in a patient comprising constructing the plant holotoxin fusion protein immunotoxin of the present invention as described above, wherein the ligand is specific for a particular cell surface receptor present on the surfaces of the cells to be targeted for intoxication and killing for the purpose of treating or preventing a cancer or autoimmune disease; and administering the plant holotoxin fusion protein immunotoxin in a pharmaceutically acceptable carrier to the patient, whereby the plant holotoxin fusion protein immunotoxin treats or prevents a cancer or autoimmune disease in the patient.

For example, the present invention provides a method of treating or preventing a cancer or an autoimmune disease in a patient comprising constructing the ricin fusion protein immunotoxin of the present invention as described above, wherein the ligand is specific for a particular cell surface receptor present on the surfaces of the cells to be targeted for intoxication and killing; and administering the ricin fusion protein immunotoxin in a pharmaceutically acceptable carrier to the patient, whereby the ricin fusion protein immunotoxin treats or prevents a cancer or autoimmune disease in the patient.

The treatment or prevention of the cancer or autoimmune disease is by the specific intoxication and killing of the cells associated, or potentially associated, with a cancer or autoimmune disease, resulting in prevention of or remission of the cancer, or prevention, elimination or reduction in severity of the symptoms of autoimmune disease. That a given plant holotoxin fusion

protein immunotoxin is effective in treating or preventing a cancer or autoimmune disease in a patient can be determined by evaluating the particular aspects of the medical history, the signs, symptoms and objective laboratory tests that have a documented utility in evaluating disease activity. These signs, symptoms and objective laboratory tests will vary depending on the particular cancer or autoimmune disease being treated or prevented as will be well known to any clinician in this field. Such methods can include, but are not limited to, x-rays, biopsies of biological samples, palpation of masses and measurements of blood and body fluid components. For example, for autoimmune disease (e.g., multiple sclerosis), clinical parameters that can be monitored can include the severity and number of attacks, or for continuously progressive disease, the worsening of symptoms and signs, the cumulative development of disability, the number or extent of brain lesions as determined by magnetic resonance imaging and the need for continued use of immunosuppressive medications (78,79).

The cancer to be treated or prevented by administration of the plant holotoxin fusion protein immunotoxin, such as, for example, ricin fusion protein immunotoxin, can be, but is not limited to, a human leukemia or lymphoma having cancer cells expressing interleukin-2 receptors on the surfaces, wherein the ligand is interleukin-2, acute myelogenous leukemia, wherein the ligand is granulocyte/macrophage-colony stimulating factor and melanoma/neuroblastoma, wherein the ligand is an antibody to GD2, as well as brain neoplasms, epithelial malignancies, sarcomas or any other cancer now known or identified in the future which can be treated or prevented by administration of the plant holotoxin fusion protein immunotoxin of the present invention. Such a cancer would express on the surface of the tumor cells or potential tumor cells an antigen to which the ligand of the present immunotoxin can selectively bind, for selective intoxication of the tumor cells or potential

tumor cells by the ricin protein immunotoxin. In identifying a tumor antigen or potential tumor antigen as a specific target antigen for the immunotoxin of this invention, one of skill in the art would examine various tumor antigens or potential tumor antigens, such as, but not limited to, growth factor receptors, adhesion molecules, oncogene products, differentiation antigens and oncofetal antigens on the surface of a subject's tumor cells or potential tumor cells.

As used herein, autoimmune disease describes a disease state or syndrome whereby a subject's body produces a dysfunctional immune response against the subject's own body components, with adverse effect. The autoimmune disease to be treated or prevented by administration of the ricin protein immunotoxin of the present invention can be, but is not limited to, graft-versus-host disease, wherein the ligand can be an antibody to CD3. Other examples of autoimmune diseases that can be treated or prevented include ulcerative colitis, Crohn's disease, multiple sclerosis, rheumatoid arthritis, diabetes mellitus, pernicious anemia, autoimmune gastritis, psoriasis, Bechet's disease, idiopathic thrombocytopenic purpura, Wegener's granulomatosis, autoimmune thyroiditis, autoimmune oophoritis, bullous pemphigoid, pemphigus, polyendocrinopathies, Still's disease, Lambert-Eaton myasthenia syndrome, myasthenia gravis, Goodposture's syndrome, autoimmune orchitis, autoimmune uveitis, systemic lupus erythematosus, Sjogren's syndrome and ankylosing spondylitis, as well as any other autoimmune disease now known or discovered in the future. Such an autoimmune disease would express an antigen on the surfaces of the cells involved or potentially involved in the autoimmune disease to which a ligand of the present fusion immunotoxin can selectively bind, in order that intoxication of the cells involved or potentially involved in the autoimmune disease can occur.

Also contemplated for the present invention is a method for treating or preventing an allergic disease in a patient comprising constructing the plant holotoxin fusion protein immunotoxin of the present invention as described above, wherein the ligand is specific for a particular cell surface receptor present on the surfaces of the cells involved or potentially involved in the allergic disease, to be targeted for intoxication and killing; and administering the plant holotoxin fusion protein immunotoxin in a pharmaceutically acceptable carrier to the patient, whereby the plant holotoxin fusion protein immunotoxin treats or prevents the patient's allergic disease. For example, the ligand can be the Fc region of immunoglobulin E (IgE).

As used herein, allergic disease describes a disease state or syndrome whereby the body produces a dysfunctional immune response composed of IgE antibodies to environmental antigens and which evoke allergic symptoms. Examples of allergic diseases include, but are not limited to, asthma, ragweed pollen hayfever, allergy to food substances and allergic reactions.

The treatment or prevention of the allergic disease is by the specific intoxication and killing of the cells associated with ,or potentially associated with, the allergic disease, resulting in the prevention of symptoms or the elimination or reduction in severity of the symptoms of the allergic disease. That a given plant holotoxin fusion protein immunotoxin is effective in treating or preventing an allergic disease in a patient can be determined by evaluating the particular aspects of the medical history, the signs, symptoms and objective laboratory test that have a documented utility in evaluating disease activity. These signs, symptoms and objective laboratory tests will vary depending on the particular allergic disease being treated or prevented as will be well known to any clinician in this field. Such methods can include, but are not limited to, x-rays, biopsies of biological samples, palpation of masses and measurements

of blood and other body fluid components. For example, clinical parameters that can be monitored for an allergic disease (e.g., asthma), can include the number and severity of attacks as determined by symptoms of wheezing, shortness of breath and coughing. The measurement of airway resistance by the use of respiratory spirometry, the extent of disability and the dependence on immunosuppressive medications or bronchodilators can also be determined (80,81).

Also contemplated for the present invention is a method of inducing immune tolerance in the patient, comprising constructing the plant holotoxin fusion protein immunotoxin of the present invention as described above, wherein the ligand is specific for a particular cell surface receptor present on the surfaces of certain immune cells to be targeted for intoxication and killing; and administering the plant holotoxin fusion protein immunotoxin in a pharmaceutically acceptable carrier to the patient, whereby the plant holotoxin fusion protein immunotoxin induces immune tolerance. For example, the ligand can be IL2 or an antibody or fragment thereof (e.g., Fv region) to the CD3 antigen.

The induction of immune tolerance is by the specific intoxication and killing of certain immune cells, resulting in the elimination or reduction in severity of a particular immune response. That a given plant holotoxin fusion protein immunotoxin is effective in inducing an immune tolerance in a patient can be determined by evaluating the particular aspects of the medical history, the signs, symptoms and objective laboratory tests that have a documented utility in evaluating immune activity. These signs, symptoms and objective laboratory tests will vary depending on the particular immune response being reduced or eliminated, as will be well known to any clinician in this field. Examples of such methods include, but are not limited to, x-rays, biopsies of

biological samples, palpation of masses and measurements of blood and other body fluid components. In particular, cytokine assays, routine clinical chemistries, immune function assays, complete blood counts and the like, as would be known to the clinician, can be measured at various intervals during treatment.

Additionally, the efficacy of administration of a particular dose of a plant holotoxin fusion protein immunotoxin in preventing a cancer, autoimmune disease, allergic disease, or immune dysfunction requiring the induction of immune tolerance (e.g., transplantation rejection) in a subject not known to have a cancer, autoimmune disease, allergic disease, or immediate need for induction of immune tolerance, but known to be at risk of developing a cancer, autoimmune disease, allergic disease or need for induction of immune tolerance, can be determined by evaluating standard signs, symptoms and objective laboratory tests, as would be known to one of skill in the art, over time. This time interval may be large, with respect to the development of cancer, autoimmune or allergic diseases (years/decades) or short (weeks/months) with respect to the development of a need for induction of immune tolerance. The determination of who would be at risk for the development of a cancer, autoimmune disease, allergic disease or in need of induction of immune tolerance would be made based on current knowledge of the known risk factors for a particular disease or immune response familiar to a clinician in this field, such as a particularly strong family history of disease or need for a transplant.

The plant holotoxins, fusion proteins and immunotoxins of the present invention are preferably provided in a pharmaceutically acceptable carrier and can be parenterally administered to the subject. Suitable carriers for parenteral administration of the immunotoxin in a sterile solution or suspension can

include sterile saline that may contain additives, such as ethyl oleate or isopropyl myristate, and can be injected, for example, intravenously, as well as into subcutaneous or intramuscular tissues.

The plant holotoxin fusion protein immunotoxin can be administered to the subject in amounts sufficient to treat or prevent a cancer, autoimmune disease or allergic disease, or to induce immune tolerance. Optimal dosages used will vary according to the individual, as well as the particular cancer or autoimmune or allergic disease being treated or the type of immune response being induced. Typically, for treatment of humans, plant holotoxin fusion protein immunotoxin, (e.g., ricin toxin fusion protein immunotoxin) would be administered intravenously in a dosage range between 1 μg and 10 mg/kg of body weight and most preferably in a dose of 0.5 mg/kg, either as a single bolus or as a continuous infusion ranging in time from a day to a month. Treatment can be continued for an indefinite period of time, as indicated by monitoring of the signs, symptoms and clinical parameters associated with a particular cancer, autoimmune disease, allergic disease or immune response induction.

The amount of plant holotoxin fusion protein immunotoxin administered will also vary among individuals on the basis of age, size, weight, condition, etc. One skilled in the art will realize that dosages are best optimized by the practicing physician and methods for determining dosage are described, for example, in *Remington's Pharmaceutical Sciences* (77). That a given dosage amount or regimen is effective in treating a cancer, autoimmune disease, or allergic disease or inducing immune tolerance, can be readily determined by using the parameters described above.

The present invention is more particularly described in the following examples which are intended as illustrative only since numerous modifications and variations therein will be apparent to those skilled in the art.

EXAMPLES

EXAMPLE I: Construction of a ricin toxin containing a modification in a lectin binding site in each of the 1α, 1β and 2γ subdomains.

Selection of RTB modifications. Connolly water-accessible surfaces of RTB subdomains 1β and 2α were plotted using Brookhaven coordinates from Rutenber and Robertus (13) and SYBYL software on a Silicon Graphics Iris Indigo workstation. Aromatic ring residues projecting into the α-carbon kink in each subdomain were identified.

Construction of transfer vectors encoding mutant RTBs. Site-specific mutagenesis was performed on single-stranded pUC119-RTB[W37S/Y248H] DNA using the Sculptor *in vitro* mutagenesis kit as previously described (40). Modifications were made at either the 1β or 2α subdomain to alter aromatic ring residues which provide van der Waals interactions between the protein and sugar. The BamHI-EcoRI mutant RTB encoding DNA fragment was then subcloned into pAcGP67A plasmid (PharMingen, San Diego, CA) and used to transform INVαF' *E. coli* cells. Transfer vectors with mutant RTBs were then purified by cesium chloride-gradient centrifugation.

Isolation of recombinant baculoviruses. pAcGP67A-mutant RTB DNAs (4 μg) were co-transfected with 0.5 μg of BaculoGold AcNPV DNA (PharMingen) into 2 x 10^6 Sf9 *Spodoptera frugiperda* insect cells as recommended by the supplier. On day 7 post-transfection, media were centrifuged and supernatants tested in limiting dilution assays with Sf9 cells as

previously described (40). Positive wells were identified and supernatants reassayed by limiting dilution until all wells up to 10^{-8} dilution were positive. Two rounds of selection were required for each mutant. Recombinant viruses in the supernatants were then amplified by infecting Sf9 cells at a multiplicity of infection (moi) of 0.1, followed by collection of day 7 supernatants.

Expression and purification of mutant RTBs. Recombinant baculoviruses were used to infect Sf9 cells at an moi of 5 in EX-CELL400 media (JRH Scientific, Lexena, KS) with 25 mM α-lactose in spinner flasks. Media supernatants and cell pellets containing mutant RTBs were collected day 6 post-infection. The supernatants were adjusted to 0.01% sodium azide, centrifuged at 3,000x g for 10 minutes, concentrated 15-fold by vacuum dialysis, recentrifuged at 3,000g for 10 minutes, dialyzed into 50 mM NaCl, 25 mM Tris (pH 8), 1 mM EDTA, 0.01% sodium azide and 25 mM α-lactose (NTEAL) at 4°C, ultracentrifuged at 100,000g for one hour at 4°C and loaded onto a P2 monoclonal antibody-acrylamide column. The column was washed with NTEAL and 500 mM NaCl, 25 mM Tris (pH 9), 1 mM EDTA, 0.01% sodium azide, 25 mM α-lactose and 0.1% Tween-20 (NTEALT). Mutant RTBs were eluted with 0.1 M triethylamine HCL (pH 11), immediately neutralized with 1 M sodium phosphate (pH 4.8) and stored at -20°C until assayed. Cell pellets were dissolved in 20 mM Tris HCL(pH 8), 50 mM NaCl, 1% NP40, 1 mM PMSF, 2 ug/ml aprotinin, 1.5 ug/ml pepstatin and 1.5 ug/ml leupeptin, frozen at -70°C, thawed, centrifuged at 22,000g for 15 minutes at 4°C , dialyzed into NTEAL at 4°C and treated identically to dialyzed concentrated cell supernatants.

Chemical and immunological characterization of mutant RTBs. Optical densities at 280 nm were determined. Aliquots were electrophoresed on a reducing 15% sodium dodecyl sulfate (SDS) polyacrylamide gel, stained

with Coomassie Blue R-250 and scanned on an IBAS automatic image analysis system. Aliquots were also electrophoresed on a reducing 15% SDS polyacrylamide gel, transferred to nitrocellulose using a Semi-dry Trans-blot cell (BioRad, Hercules, CA), and immunoblots were performed with rabbit anti-ricin antibody as previously described (40). Antibody enzyme linked immunosorbent assays (ELISA), using monoclonal anti-RTB antibodies P2, P8 or P10 as well coats, were done on each mutant following the method previously reported (15,40).

Lectin activity of mutant RTBs. Asialofetuin ELISAs and experiments to analyze binding to KB cells in the presence or absence of 100 mM α-lactose or 100 ug/ml asialofetuin were performed as previously described (15).

Reassociation of mutant RTBs with plant RTA to form heterodimers. 0.25 ml of mutant RTBs (5 - 15 µg) was mixed with a 4 - fold molar excess of plant RTA (Inland Laboratories) in 0.1 M triethylamine-sodium phosphate pH 7 overnight at room temperature. The reaction mixture was then analyzed by a ricin ELISA utilizing P2 monoclonal anti-RTB coated wells, biotin conjugated αBR12 monoclonal anti-RTA and alkaline phosphatase-conjugated streptavidin detection reagents, as previously described (15).

Cytotoxicity assays. HUT102 cells were incubated with dilutions of ricin and mutant RTB-plant RTA heterodimers at varying concentrations for 24 hours and pulsed for four hours with ^{3}H-leucine as previously detailed (15,40). The 50% inhibitory concentration (IC_{50}) for ricin and each mutant heterodimer was calculated as the concentration which inhibited protein synthesis by 50% as compared with control.

Aromatic ring residues in lectin pocket of subdomains 1β and 2α. The aromatic amino acid residues projecting into the binding cleft were identified to be Tyr-78 and Trp-160. These two residues were selected for mutagenesis to histidine and serine, respectively.

Yields and immunoreactivity of mutant RTBs. Yields were estimated from the optical density at 280 nm of neutralized alkaline eluants post-affinity chromatography (plant RTB OD = 1.44 for 1 mg/ml) and densitometry of Coomassie-stained reducing SDS polyacrylamide gels (10-30% of the protein migrated at 33 kilodaltons). Results were confirmed by densitometry of immunoblots reacted with rabbit anti-ricin antibody. Both triple-site mutants were reactive with the polyclonal antibody. Finally, a monoclonal antibody anti-RTB ELISA was used to verify concentrations of each mutant. All three assays gave similar values. The yield from cell supernatants of the parent double-site mutant, W37S/Y248H, was 205 ug/liter of Sf9 culture. The yield of triple-site mutant, W37S/Y248H/Y78H was 750 ug/liter of culture and the yield of triple-site mutant, W37S/Y248H/W160S was 180 ug/liter of culture. Yields from cell extracts were similar to yields from supernatants for all three mutants.

Reactivities of the mutant RTBs with different monoclonal antibodies to RTB (P2, P8 and P10) were tested by substituting different monoclonal antibodies as capture reagents in the antibody ELISA. Equivalent results were observed for each antibody suggesting similar folding of the mutants. W37S/Y248H RTB reacted 1.3-fold and 4.0-fold more with P8 and P10 monoclonal antibodies, relative to P2 antibody. W37S/Y248H/Y78 RTB reacted 0.7-fold and 1.8-fold as well with P8 and P10 antibodies as with P2 antibody. Finally, W37S/Y248H/W160S RTB bound 1.0-fold and 2.3-fold to P8 and P10, respectively, relative to P2.

Excellent yields of triple-site mutants. The recovery of 0.18 and 0.75 mg triple-site mutant RTB/liter from infected Sf9 cell supernatants was higher than the yields of six single-site RTB mutants and four double-site RTB mutants (15). Further, similar quantities of triple-site mutants were recovered from cell pellets--440 ug/liter for W37S/Y248H/Y78H RTB and 70 ug/liter for W37S/Y248H/W160S RTB. The yields were not dissimilar from the yields for the double-site mutant W37S/Y248H (220 ug/liter from supernatant and 250 ug/liter from cell pellet) or wild-type RTB (400 ug/liter for supernatants) in this expression system and may reflect proper folding for the triple-site mutants. The conservative modification of surface residues (Trp to serine and Tyr to histidine) may have contributed to protein stability.

Immunoreactivity of triple-site mutants. Both triple-site mutants reacted with all monoclonal and polyclonal anti-RTB antibodies tested. Further evidence that these RTB mutants were properly folded included their stability at 4°C and -20°C for one month in 0.1 M triethylamine/sodium phosphate pH 8 and their ability to reassociate with plant RTA.

Sugar binding of mutant RTBs. The double-site RTB mutant, W37S/Y248H, bound asialofetuin 4.8 $\pm$ 2% (n = 6) relative to recombinant or plant RTB. The triple-site RTB mutant, W37S/Y248H/W160S, bound asialofetuin similarly at 1.1 $\pm$ 0.27% (n = 7) relative to plant RTB. In contrast, the triple-site mutant, W37S/Y248H/Y78H, showed minimal to negligible binding to asialofetuin at 0.2 $\pm$ 0.08% (n = 7), relative to plant RTB which was two to three fold higher than background.

An independent measure of mutant RTB binding to glycoproteins was made by detecting mutant RTB bound to cell surfaces. Only W37S/Y248H and W37S/Y248H/W160S showed significant binding to KB cells at 4°C.

Sugar binding of triple-site mutants. W37S/Y248H/W160S RTB retained binding to immobilized asialofetuin and KB cell surface glycoproteins. In both cases, the binding was competed with soluble saccharides. In contrast, W37S/Y248H/Y78H had minimal to negligible sugar binding. These results differ from the findings of Yen and Vitetta with Cos cell-derived mutant RTB (43), Wales and colleagues with Xenopus laevis cell-derived RTBs (44) and Swimmer and colleagues with bacteriophage gene III fusion proteins (45). They reported complete inactivation of sugar binding by modifications of residues in a single subdomain (43) or two subdomains (44,45). However, very small amounts of protein were made and no purification or immunological characterization of the products were done. In each case, decreased sugar binding may have been due, in part, to misfolding or aggregation of recombinant RTBs leading to an overestimation of the effect of their modifications.

Competition experiments. Binding of W37S/Y248H to immobilized asialofetuin was inhibited 3-fold by 100 mM α-lactose and 5-fold by 100 ug/ml asialofetuin. Similarly, W37S/Y248H/W160S binding was inhibited 9-fold by lactose and 27-fold by asialofetuin. Binding of the triple-site mutant W37S/Y248H/Y78H was minimally inhibited by either lactose or asialofetuin--3-fold in each case. Binding of the double-site mutant and 1α, 2α, 2γ triple-site mutant to KB cells was blocked by 100 ug/ml asialofetuin.

Heterodimer formation. Incubation of 5×10^{-7} M - 2.5×10^{-6} M mutant RTBs with excess plant RTA overnight at room temperature led to 70% reassociation of W37S/Y248H, 24% reassociation of W37S/Y248H/Y78H and 57% reassociation of W37S/Y248H/W160S. Similar levels of reassociation were seen using plant RTB or recombinant wild-type RTB with plant RTA under the same conditions. The heterodimer concentrations were quantitated

by an ELISA which identified molecules with both RTB and RTA epitopes and by densitometry of 65 kilodalton bands of immunoblots with anti-RTB and anti-RTA antibodies. Both ELISA and immunoblots gave similar values and showed both mutants reassociated well with plant RTA and had minimal homodimer formation.

Cytotoxicity of mutant heterodimers. The IC_{50} of ricin on HUT102 human leukemia cells was 4×10^{-12} M. The IC_{50} for W37S/Y248H was 2×10^{-10} M; the IC_{50} for W37S/Y248H/W160S was 1×10^{-10} M; and the IC_{50} for W37S/Y248H/Y78H was 5×10^{-9} M. Plant RTA alone had a 20-fold higher IC_{50} of 10^{-7} M.

Cell intoxication functions of the triple-site mutants. Cell sensitivity to mutant heterodimers paralleled mutant residual sugar-binding activity. Significant residual potency was seen with the 1α,2γ and 1α,2α,2γ mutant heterodimers. Their IC_{50}'s were at least two and one-half logs lower than plant RTA alone (RTB alone was nontoxic with $IC_{50} > 6 \times 10^{-6}$M). In contrast, the 1α,1β,2γ mutant RTB-RTA had minimal toxicity above background.

Widely separated ricin lectin sites. The 1α and 2γ sites are separated by 36 Angstroms. 1β and 2γ sites are 44 Angstroms apart. The 1α and 1β distance is 19 Angstroms. These inter-binding site distances are much larger than the inter-site spacing for the hepatic Gal/GalNac receptor and its triantennary N-glycoside ligand (10 -20 Angstroms) (41). Instead, the ricin geometry resembles the spacing of sites on surface binding lectins including mammalian mannose-binding protein, influenza virus hemagglutinin, pertussis toxin and cholera toxin (42). These proteins are phylogenetically unrelated based on lack of primary or tertiary structure homology. Nevertheless, in all these proteins, the sugar combining sites are multiple widely spaced and project

toward a single plane. Thus, they are ideally suited for binding to eukaryotic cell surfaces.

Three RTB lectin sites. The three binding sites on ricin may provide the optimal geometry for binding to the uneven galactosyl oligosaccharide-rich surface of mammalian cells similar to camera tripods or stools. The RTB 1γ and 2β subdomains are unlikely to contribute additional sugar binding as they lack the tripeptide α-carbon kink, aromatic residues or charged residues for hydrogen bond formation. Further, no RGD-like domains exist in RTB--unlike discoidin I from the slime mold, *Dictylostelium discoideum* (46).

EXAMPLE II: *In vivo* toxicity of a ricin toxin containing a modification in a lectin binding site in each of the 1α, 1β and 2γ subdomains.

Ricin and recombinant heterodimers. Purified castor bean ricin (3.9 mg/ml in 0.15 M NaCl, 0.015 M potassium phosphate pH 7, 0.1% sodium azide) was purchased from Sigma (St. Louis, MO). Purified deglycosylated RTA (5 mg/ml in PBS) was a gift of Dr. Jerry Fulton, Inland Laboratories, Dallas, Texas. Partially purified recombinant mutant RTBs were prepared and reassociated with plant RTA as previously described (15,40). The proteins tested and their characteristics are shown in Table 1. Ricin was stored at 4°C and all other tested proteins were stored at -20°C until used.

Mice. Pathogen-free C57B/6 female mice (16-18 grams) were purchased from Jackson Laboratories (Bar Harbor, ME) or Harlan Sprague-Dawley (Indianapolis, IN), culled on each experiment to obtain 24 animals each 18 ± 1 grams, and housed in groups of four in specific pathogen-free environment in Micro-Isolator cages (Lab Products, Maywood, NY).

Experimental Design. Mice were injected intraperitoneally with dilutions of toxins in phosphate buffered saline (PBS) plus 0.5% bovine serum albumin (BSA) so that each animal received 0.25 - 0.5 milliliters solution. Animals were then observed twice daily for mortality. Six different concentrations were tested on groups of four mice each for each protein. Graphs of animal survival versus time were prepared for each protein. Once the LD_{50} was determined for each protein, single animals received the LD_{50} and were collected at death or when severely morbid and autopsies were performed.

Chi square contingency tables were formulated for groups of doses for each toxin as described (47).

Histology protocols. Postmortem examinations were carried out on mice given LD_{50} doses on moribund animals. Samples from lungs, thymus, heart, esophagus, trachea, lymph node, liver, spleen, pancreas, kidneys, adrenals, gallbladder, stomach, duodenum, jejunum, colon, ovaries, uterus, brain, spinal cord, and skeletal muscle were taken for microscopic examination. The tissues were fixed in 4% buffered formaldehyde and embedded in paraffin. Sections were stained with hematoxylin and eosin.

Ricin wild-type, mutants, and subunits. The ricin proteins tested for *in vivo* toxicity are listed in Table 1. Examples of wild-type ricin and ricins with modifications of one, two or three sites were used. The insect-derived RTB mutants had similar folding to plant RTB based on yield from insect cells, migration on SDS-PAGE, immunoreactivities with polyclonal and monoclonal antibodies, CD spectra, protease sensitivity and ability to reassociate efficiently with plant RTA, as described above. The concentration of recombinant heterodimers was based on a sandwich ELISA previously described (15).

Mouse lethality of ricin proteins. Castor bean ricin led to death of all four mice receiving 500 ng three days post-injection. Three of four mice receiving 100 ng died on days 5, 7 and 8 post-injection. None of the mice receiving 1, 5, 10 or 50 ng ricin died. Thus, higher doses led to earlier animal deaths. The LD_{50} was estimated as 75 ng.

Mice treated with 130 ng, 650 ng, 1.3 μg, or 2.6 μg all died after injection with RTB[Y248H]-RTA (2γ single-site mutant heterodimer). Three of four mice treated with 2.6 μg died on day 2 and one of four mice died on day 4 post-injection. Three of four mice treated with 1.3 μg died on day 3 and one of four died on day 5. One of four mice receiving 650 ng died on day 4 and the rest died on day 5. The 130 ng treated mice died on day 5 (1 mouse), day 6 (2 mice) and day 7 (1 mouse). Three of four mice treated with 260 ng died on day 6 (one mouse) and day 7 (two mice). None of the mice treated with 65 ng died. Similar to wild-type ricin, higher doses led to animal deaths on day 3 and 4 post-injection, while the doses closer to the LD_{50} caused death on days 5 - 7. The estimated LD_{50} was 100 ng.

Mice given RTB[W37S/Y248H]-RTA (1α/2γ double-site mutant heterodimer) tolerated more toxin than animals receiving wild-type or single-site mutant ricin. One of four animals died on day 1 and 3/4 animals died on day 2 post-injection with 10 μg. Three of four animals died on day 1 post-injection of 5 μg. Three of four animals died on day 2 and one of four died on day 3 post-2 μg injections. The 1 μg dose killed two mice--one each on day 4 and 5. Similarly, the 500 ng dose led to the death of two mice--one each on days 7 and 10. None of the mice receiving 100 ng died. Again, animals treated with doses much higher than the LD_{50} died on days 1-3, while animals treated with lower toxic doses died on days 4 - 10. The LD_{50} was estimated at 500 ng.

Only limited toxicity was observed with RTB[W37S/Y248H/Y78H]-RTA (1α/1β/2γ triple-site mutant heterodimer). None of the mice receiving 0.1 μg, 0.5 μg, 1 μg, 2 μg, or 5 μg died and only one of four mice receiving 10 μg died on day 3 post-injection. Thus, the LD_{50} was estimated at >10 micrograms.

Plant RTA was significantly less toxic than either the wild-type ricin or mutant heterodimers.

Using chi square contingency tables, the survival rates of mice treated with RTA and the triple-site mutant ricin were calculated to be significantly different ($p<0.001$) than the survival rates of wild-type, single-site mutant or double-site mutant ricins over a range of doses.

The relationship of time to death post-toxin infusion with the ratio dose/LD_{50} for the ricin proteins was also examined. A linear regression analysis showed the correlation was of borderline significance with an $r^2 = 0.48$. Previous investigators have demonstrated earlier deaths at higher toxin doses (52).

The relationship of toxic lectin Kd or dissociation constant for asialofetuin binding with the LD_{50} was also analyzed. A striking correlation was seen with an r^2 of 0.959. Thus, over 95% of the variation in LD_{50} could be explained by sugar binding avidity.

Histology results. The mice treated with intraperitoneal ricin or RTA showed apoptosis in the thymus and spleen. Peritoneal inflammation was observed in some animals presumably due to intraperitoneal injection of toxin. There was no evidence of apoptosis based on nuclear morphology in the other

organs examined. The triple-site mutant heterodimer treated mouse showed no histopathologic abnormalities.

The critical target tissue of ricin in animals is unknown. Previous histopathologic examination similarly failed to reveal differences from untreated animals at either the light or electron microscopic level (8). Protection of mice by intracerebral injection of anti-ricin antibodies led to the hypothesis that the brain is the lethal target for ricin (48), however very little radiolabeled ricin was shown to cross the blood-brain barrier and reach the central nervous system (49). No evidence of central nervous system damage was found in this histological study. Nevertheless, the observation in the present study that modification of lectin sites reduces *in vivo* toxicity supports the role for galactoside receptors on cells in the critical target organ.

The finding of lymphoid apoptosis after ricin administration might suggest that those cells have a lower apoptotic threshold. Deletion of lymphocytes is an important ongoing process for tolerance induction and immune regulation in the lymphoid system (50).

The toxicity of RTA alone may be due to the presence of small amounts (<0.1%) of contaminating ricin. Histopathology in this study did not show extensive renal injury previously reported by others with membranous glomerulopathy, periglomerular fibrosis and tubular hyaline casts (51). In the study by Soler-Rodriguez, hypoalbuminemia and weight gain were also demonstrated. These findings were compatible with a vascular endothelial injury. However, reproducible histologic evidence of endothelial or vascular damage was not demonstrated in this study.

The effect of modification of a third RTB subdomain on ricin toxicity was dramatic with a > 20-fold increase in LD_{50}.

EXAMPLE III: Construction of a ricin fusion protein immunotoxin containing a modification in a lectin binding site in each of the 1α, 1β and 2γ subdomains.

Construction of plasmid. Site-specific mutagenesis was performed on single-stranded pUC119-RTB[W37S/Y248H] DNA using the Sculptor *in vitro* mutagenesis kit (Amersham, Arlington Heights, IL) as previously described (40). The aromatic ring residue Tyr-78 in the 1β subdomain was changed to histidine to reduce van der Waals interactions between the protein and galactosides. The BamHI-EcoRI mutant RTB encoding DNA fragment was subcloned into pAcGP67A plasmid (PharMingen, San Diego, CA) and used to transform INVαF' *E. coli* cells (InVitrogen, San Diego, CA). Transfer vector with mutant RTB was then purified by cesium chloride density centrifugation, restricted with BamHI, bound and eluted from silica matrix (Promega, Madison, WI), digested with calf intestinal phosphatase (Boehringer-Mannheim, Indianapolis, IN), heat inactivated and repurified on silica matrix. The BamHI fragment encoding IL2 prepared by polymerase chain reaction of pDW27 plasmid DNA as previously described (54) was isolated from pUC119-IL2 by digestion of cesium chloride density gradient purified plasmid with BamHI, agarose electrophoresis and binding and elution from silica matrix. The 406 bp fragment was subcloned into pAcGP67A-ADP-RTB[W37S/Y248H/Y78H]. The expression vector was maintained in INVαF' *E. coli* using 100 ug/ml ampicillin. Plasmid isolated by alkaline lysis followed by cesium chloride density gradient centrifugation was double-stranded dideoxy sequenced by the Sanger method (33) using the Sequenase kit (USB, Cleveland, OH).

Expression of fusion toxin. Sf9 *Spodoptera frugiperda* ovarian cells (2 x 10^6), maintained in TMNFH medium supplemented with 10% fetal calf serum and 50 ug/ml gentamicin sulfate, were co-transfected with pAcGP67A-ADP-IL2-ADP-RTB[W37S/Y248H/Y78H] DNA (4 μg) and 0.5 μg of BaculoGold AcNPV DNA (PharMingen) following the recommendations of the supplier. At 7 days post-transfection, medium was centrifuged and the supernatant tested in a limiting dilution assay with Sf9 cells and dot blots with random primer ^{32}P-dCTP labeled RTB DNA as previously described (53). Positive wells were identified and supernatants reassayed by limiting dilution until all wells up to 10^{-8} dilution were positive. Two rounds of selection were required. Recombinant virus in the supernatant was then amplified by infection Sf9 cells at an moi of 0.1, followed by collection of day 7 supernatants. Recombinant baculovirus was then used to infect 2 x 10^8 Sf9 cells at an moi of 5-10 in 150 ml EXCELL400 medium (JRH Scientific, Lexena, KS) with 25 mM lactose in spinner flasks. Media supernatants containing ADP-IL2-ADP-RTB[W37S/Y248H/Y78H] were collected at day 6 post-infection. Three different preparations were made.

Protein purification. Media supernatants were adjusted to 0.01% sodium azide and maintained through all purification steps at 4°C. The supernatants were concentrated 15-fold by vacuum dialysis, centrifuged at 3,000x g for 10 minutes to remove precipitate, dialyzed against 50 mM NaCl, 25 mM Tris (pH 8), 1 mM EDTA, 0.01% sodium azide and 25 mM lactose (NTEAL), ultracentrifuged at 100,000g for one hour and bound and eluted from a P2 monoclonal antibody-acrylamide matrix as previously described (53). P2 is an anti-RTB monoclonal antibody. The affinity matrix was prepared using Ultralink azlactone functionality bis-acrylamide following the recommendations of the manufacturer (Pierce, Rockford, IL). Recombinant protein was absorbed to the column in NTEAL, washed with 0.5 M NaCl, 25

mM Tris pH 9, 1 mM EDTA, 0.1% Tween 20, 0.02% sodium azide, 25 mM lactose and eluted with 0.1 M triethylamine hydrochloride (pH 11). The eluant was neutralized with 1/10 volume 1 M sodium phosphate pH 4.25 and stored at -20°C until assayed. Three preparations were made.

Characterization of recombinant protein. Total protein concentration of the affinity column eluant was measured by absorbance at 280 nm. Since the optical densities of a 1 mg/ml solution of RTB and IL2 were 1.4 and 0.7, respectively, a 1 mg/ml solution of fusion protein should have a mass average optical density of 1.16. Protein was also quantitated by BioRad protein assay as per recommendations of the supplier. Aliquots of ADP-IL2-ADP-RTB[W37S/Y248H/Y78H], plant RTB and prestained low molecular weight standards were electrophoresed on a reducing 15% SDS polyacrylamide gel, stained with Coomassie Blue R-250 and scanned on an IBAS automatic image analysis system (Kontron, Germany). Immunological analysis was performed using both an ELISA and immunoblot format. Costar EIA microtiter wells were coated with 100 µl of 5 ug/ml of monoclonal antibody P2, P8, or P10 reactive with RTB or monoclonal antibody to IL2, washed with PBS plus 0.1% Tween 20, blocked with 3% BSA, rewashed and incubated with samples of ADP-IL2-ADP-RTB[W37S/Y248H/Y78H], human IL2 or plant RTB, rewashed, reacted with 1:400 rabbit antibody to ricin or 1:500 rabbit antibody to IL2, washed again, incubated with 1:5000 alkaline phosphatase conjugated goat anti-(rabbit IgG), rewashed, developed with 1 mg/ml p-nitrophenylphosphate in diethanolamine buffer (pH 9.6) and read on a BioRad 450 Microplate reader at 405 nm. Aliquots of ADP-IL2-ADP-RTB[W37S/Y248H/Y78H], bacterial IL2, recombinant RTB, plant RTB, and prestained low molecular weight protein standards were run on a reducing 15% SDS-PAGE, transferred to nitrocellulose, blocked with 10% Carnation's nonfat dry milk/0.1% bovine serum albumin (BSA)/0.1% Tween 20, washed with PBS

plus 0.05% Tween 20, reacted with either 1:400 rabbit antibody to ricin or 1:100 mouse monoclonal antibody to IL2 (5 ug/ml), rewashed, incubated with alkaline phosphatase conjugated goat anti-(rabbit IgG) or anti-(mouse IgG), washed again and developed with the Vectastain alkaline phosphatase kit.

RTA Reassociation protocols. 30 µg of ADP-IL2-ADP-RTB[W37S/Y248H/Y78H] was mixed with 100 µg of plant RTA in a total volume of 0.5 ml of 0.1 M triethylamine/0.1 M sodium phosphate (pH 7) and shaken overnight at room temperature. The reaction mixture was then analyzed by a modified ricin ELISA as previously described (15). Reassociated mixtures were also analyzed by non-reducing SDS/PAGE followed by immunoblots with P2 and P10 anti-RTB monoclonal antibodies (10 ug/ml each), monoclonal antibody to IL2 (5 ug/ml) or monoclonal antibody αBR12 to RTA (10 ug/ml) . Densitometric scanning with the automatic image analysis system was done to quantify the shift of immunoreactive material from 50 kDa to 80 kDa.

Lectin activity assay. Asialofetuin (1 ug/ml) was bound to Costar EIA plate wells and an ELISA was performed as previously detailed with samples of ADP-IL2-ADP-RTB[W37S/Y248H/Y78H]-RTA and castor bean ricin (15) Briefly, the asialofetuin coated wells were washed with PBS plus 0.1% Tween 20, blocked with 3% BSA, rewashed and incubated with 12 different concentrations of samples in EX-CELL400, rewashed and reacted with 100 µl of biotinylated αBR12 monoclonal anti-RTA antibody, rewashed and incubated with streptavidin-alkaline phosphatase, washed again and developed with p-nitrophenylphosphate in 50 mM diethanolamine (pH 9.6). Absorbance of wells was measured at 405 nm on a microtiter plate reader. The concentration of protein giving half-maximal binding (Kd) was calculated.

IL2 receptor binding specificity assay. HUT102 human T leukemia cells bearing the high affinity IL2 receptor, YT2C2 human leukemia cells bearing the intermediate affinity IL2 receptor, MT-1 human leukemia cells bearing the low affinity IL2 receptor and CEM human leukemia cells and KB human epidermoid carcinoma cells lacking the IL2 receptor were washed with PBS and attached to polylysine-coated tissue culture dishes and centrifuged at 2000g for 10 minutes. The IL2R content of these cells was previously determined (15). The cells were then incubated live at 4°C. The cells were washed with 2 mg/ml BSA in PBS and incubated in PBS plus BSA with 1 ug/ml castor bean ricin or IL2-lectin-deficient-ricin. The incubation was done at 4°C. The cells were then washed with PBS and incubated with αBR12 mouse monoclonal antibody to RTA (5 ug/ml) plus BSA for 30 minutes at 4°C. The cells were then washed with PBS and reacted with goat anti-(mouse Ig) conjugated to rhodamine (Jackson ImmunoResearch, West Grove, PA) at 25 ug/ml for 30 minutes at 4°C. The cells were washed again in PBS and fixed in 3.7% formaldehyde in PBS, mounted under a #1 coverslip in glycerol-PBS (90:10) and examined using a Zeiss Axioplan epifluorescence microscope.

Cytotoxicity assay. Measurement of protein synthesis inhibition by ricin and ADP-IL2-ADP-RTB[W37S/Y248H/Y78H]-RTA in cultured cells was done as previously described using HUT102, CEM, YT2C2, MT-1, and KB cells (15). ADP-IL2-ADP-wild-type RTB-RTA and ADP-IL2-ADP-RTB[W37S/Y248H] prepared as described previously were also tested.[18,22] All assays were performed in triplicate. Twelve different concentrations of toxins were used. The ID_{50} was the concentration of protein which inhibited protein synthesis by 50% compared with control wells without toxin. There was no purification step after heterodimer reassociation. The free RTA concentration at the highest concentration of heterodimer in the assay (5×10^{-7}M) was 10^{-7}M. On all five cell lines, the ID_{50} for free RTA was $2 - 3 \times 10^{-6}$ M.[28] Thus, in the

range of heterodimer ID_{50}'s (10^{-8} M - 10^{-12} M), the free RTA concentration (2 x 10^{-9} M - 2 x 10^{-13} M) should not produce cytotoxicity.

Blocking of cytotoxicity with IL2 or lactose. HUT102 cells (1.5×10^4) were placed in sterile Eppendorf tubes at 4°C in 100 µl leucine-poor RPMI 1640 + 10% dialyzed fetal calf serum with or without 20 ug/ml IL2 or 60 mM α-lactose. Dilutions of IL2-lectin-site modified ricin and ricin at varying concentrations were added in identical medium with or without IL2 or lactose and incubated at 4°C for 30 minutes. Cells were pelleted at 2000g for five minutes, washed once with leucine-poor RPMI 1640 + 10% dialyzed fetal calf serum, resuspended in 150 µl of the same medium and incubated at 37°C in 5% CO_2 for 24 hours. ^{3}H-leucine was added as above and cells were harvested four hours later with a Skatron cell harvester and ^{3}H-leucine incorporation was measured in a liquid scintillation counter. Blocking of selective cytotoxicity was estimated by comparing the ID_{50} of toxins in the presence or absence of IL2 or lactose.

Yield and purity of ADP-IL2-ADP-RTB[W37S/Y248H/Y78H]. Three individual preparations from 100 ml cell supernatants were partially purified. Peak eluant fractions contained 186 µg, 228 µg and 167 µg protein based on absorbance at 280 nm. BioRad protein assay gave values of 120 µg, 160 µg, and 140 µg, respectively for the three preparations, using a BSA standard. Densitometry of Coomassie stained gels showed only a single detectable band at 50 kDa in each preparation. However, P2 antibody ELISA showed the concentration of anti-RTB immunoreactive protein was 102 µg, 49 µg, and 75 µg, respectively. Thus, purity was between 21 and 85 % based on absorbance, BioRad protein assay and densitometry of Coomassie-stained gels.

Immunologic cross-reactivity of ADP-IL2-ADP-RTB[W37S/Y248H/Y78H]. Different monoclonal antibodies to RTB (P2, P8 and P10) and an antibody to IL2 reacted similarly with the fusion molecule based on antibody capture ELISA. The concentration of the fusion molecule was based on a comparison of P2 antibody binding with plant RTB, so that its relative binding is taken as 100%. Antibody P8 bound 100 % as well with the fusion molecule as with plant RTB. Antibody P10 bound 500 % as well with IL2-lectin deficient-RTB as with plant RTB. Antibody to IL2 bound the hybrid molecule 20% as well as with recombinant human IL2 on a molar basis.

Immunoblots demonstrated reactivity with the same 52 kDa band using anti-RTB or anti-IL2 antibodies. No weaker bands at lower molecular weight were observed with either set of antibodies, suggesting the partial proteolysis found with IL2-"wild-type" RTB was not present with the lectin-deficient chimeras.

Reassociation with RTA. Two preparations of fusion toxin heterodimer were made. Under the reaction conditions (10^{-6} M of IL2-lectin-deficient RTB and 3 x 10^{-6} M of plant RTA, 0.1 M triethylamine/0.1 M sodium phosphate pH7, room temperature, room air), 83% reassociation was observed in one reaction and 78 % reassociation was seen in the other. The results from the sandwich ricin ELISA were confirmed by immunoblots with antibodies to RTB, RTA and IL2.

Lectin activity and IL2R binding of the heterodimer. ADP-IL2-ADP-RTB[W37S/Y248H/Y78H]-RTA bound immobilized asialofetuin 0.3% as well as plant ricin. The ricin Kd was 4 x 10^{-9} M and the IL2 fusion toxin Kd was 1.2 x 10^{-7} M. Specificity for high affinity IL2 receptor was demonstrated on a live cell immunofluorescence assay. The IL2 fusion toxin bound to HUT102,

YT2C2, MT-1 but did not bind CEM or KB cells. Binding to HUT102 cells was inhibited by IL2 but not asialofetuin.

Cell cytotoxicity to mammalian cells. Cytotoxicities of fusion heterodimer and plant ricin for different cell lines is shown in Table 2. Ricin was uniformly toxic to all five cell lines tested with IC_{50} values of 2 - 3.5 x 10^{-12} M. IL2-wild-type RTB-RTA was also toxic to all cell lines in the absence of lactose with IC_{50} values of 2 -4 x 10^{-12} M. IL2-RTB[W37S/Y248H]-RTA showed moderate specificity with IC_{50} values of 4 x 10^{-12} M on HUT102 cells, 1.8 x 10^{-10} M on CEM cells and 2 x 10^{-10} M on KB cells. In contrast, the IL2-triple-site RTB mutant-RTA had improved specificity with IC_{50} values of 5 x 10^{-12} M on HUT102 cells, 1 x 10^{-9} M on CEM cells and 6 x 10^{-10} M on KB cells. The *in vitro* therapeutic window (the ratio of the IC_{50} of receptor negative cells to the IC_{50} of receptor positive cells) was 1 for IL2-wild-type RTB-RTA, 50 for IL2-RTB[W37S/Y248H]-RTA and 120 - 200 for IL2-RTB[W37S/Y248H/Y78H]-RTA.

IL2 receptor-mediated cell toxicity was tested by blocking experiments with excess IL2 or lactose. Excess IL2 reduced IL2-triple-site RTB mutant-RTA toxicity towards HUT102 cells by 1,000-fold (IC_{50} was 1 x 10^{-8} M with IL2 and 1 x 10^{-11} M without IL2). In contrast, excess lactose had minimal effect on IL2-lectin-deficient ricin cytotoxicity (IC_{50} was 1.6 x 10^{-11} M with lactose and 1.4 x 10^{-11} M without lactose).

Synthesis of an IL2R targeted ricin fusion protein for preclinical and clinical development requires adequate yields, simple purification, adequate stability at room temperature and 37°C, and selective toxicity to IL2R bearing lymphocytes.

The IL2 triple-site mutant RTB fusion protein was obtained at 50% purity in good yields of 0.75 mg/liter culture. This compares with 1 mg/liter for IL2 wild-type RTB and 0.34 mg/liter IL2 double-site mutant RTB fusion protein. The IL2 triple-site mutant RTB molecules reacted with antibodies to IL2 and RTB both by ELISA and Western blots. These results are evidence of proper folding of both the IL2 and RTB domains. Further, the protein was secreted into the insect cell medium, and purification was accomplished by a one-step immunoaffinity absorption. This contrasts with the requirement for denaturation and refolding for bacterial toxin fusion proteins and chemical derivatization and conjugation for immunotoxins.

The toxophore domain of ricin (RTA) was added by simply mixing with the IL2 triple-site mutant RTB at 10^{-6} M. Extensive ionic and hydrophobic bonds in the RTA-RTB interface promote reassociation and disulfide bond formation (13). The observation of 80% reassociation compares favorably with the 60% reassociation for IL2 wild-type RTB-RTA (54), 55% reassociation for IL2 double-site mutant RTB-RTA and 50% reassociation for plant RTB-RTA under identical conditions (53). The heterodimers were stable at high dilution (10-12 M) suggesting formation of the disulfide bond between RTA Cys-259 and RTB Cys-4 (55).

Binding specificity of the lectin-deficient heterodimer was demonstrated in both ELISA and cell immunofluorescence formats. The fusion toxin displayed 0.3% binding to immobilized asialofetuin. The Kd was 1.2×10^{-7} M versus 4×10^{-9} M for plant ricin. This weak binding compares to 1% binding (Kd = 4×10^{-7} M) for IL2-RTB[W37S/Y248H]-RTA and 59% binding (Kd = 7×10^{-9} M) for IL2-wild type RTB-RTA. The low level binding observed in the ELISA is near the limits of detection in this assay (lower limit 0.1% relative to wild type ricin or Kd = 4×10^{-6} M). Nevertheless, the small residual binding of

the 1α, 1β, 2γ triple mutant fused to IL2 appears to be real and due to incomplete inactivation of one or more sites. Subdomain 1α mutation W37S reduced sugar binding avidity 4-fold, while other 1α subdomain mutations (K40M and K40M/N46G) yielded proteins with 7-8-fold reductions in asialofetuin avidity (40). The W37S mutation was used in the IL2-triple-site mutant because of its much better yields.

IL2 triple-site RTB mutant-RTA bound to cells possessing low, intermediate and high affinity IL2R. The lack of detectable binding to other human cell lines which still have cell surface galactosides may be due to the insensitivity of the immunofluorescence assay. a two and one-half log reduction in sugar binding may be beneath the immunofluorescence detection limit. Binding to cell lines was blocked by IL2 but not asialofetuin.

The most important property of an IL2-lectin-deficient ricin fusion protein is its selective cytotoxicity to IL2R expressing cells. Three previous studies suggested that targeted ricin molecules in which the galactose-binding sites were removed genetically or chemically lose critical intracellular intoxication functions and cannot kill cells (33-35). Goldmacher used antibody conjugates to ricin with one, two or three affinity cross-linkers (33). The triply cross-linked molecules lacked sugar-binding and were unable to intoxicate antigen-bearing cells. Newton reassociated *Xenopus laevis* oocyte-derived RTB with modifications of two lectin sites (K40M/N46G/N255G) with plant RTA (34). The lectin-deficient heterodimer was nontoxic to mouse macrophages in the presence of lactose, even though binding and internalization was mediated by binding to mannose receptors. Finally, Youle attached mannose-6-phosphate to tyrosyl acetylated ricin and measured cytotoxicity to mannose-6-phosphate receptor expressing fibroblasts in the presence of lactose (35). The lectin-deficient ricin conjugate again had reduced

cell cytotoxicity, although cell binding and entry was mediated by non-galactoside mechanisms.

The IL2-lectin-deficient fusion toxin was selectively cytotoxic to hematopoietic neoplastic cell lines with the heterotrimeric high affinity IL2R. The molecules were less toxic to cells with intermediate or low affinity IL2R, and nontoxic to cells without IL2R. These results are similar to those seen with IL2-PE40[35] but distinct from those seen with DAB_{389}IL2. The latter molecule fails to intoxicate low affinity IL2Rα,γ cells perhaps due to steric effects of the N-terminal toxin moiety. As a note of caution, fresh leukemic blasts often display lower levels of IL2Rα and IL2Rβ than cell lines and may show lowered sensitivity to the ricin fusion molecule (23).

Competition experiments with excess ligand demonstrated that IL2, but not lactose, inhibited cell cytotoxicity of the IL2-triple-site RTB mutant-RTA. Thus, the fusion toxin needs IL2R binding for cell intoxication.

EXAMPLE IV: Studies of mannose receptor mediated cell cytotoxicity of ricin fusion protein immunotoxins containing a modification in a lectin binding site in each of the 1α, 1β and 2γ subdomains.

Preparation of toxins. Recombinant baculovirus encoding the gp67A leader and the RTB mutant (W37S/Y78H/Y248H) was prepared as previously described and used to infect Sf9 insect cells at an moi of 5-10 in EXCELL400 medium (JRH Scientific, Lexena, KS) supplemented with 25 mM lactose. W37SiY78H/Y248H RTB protein was purified from day 6 post-infection cell supernatants by, sequentially, vacuum dialysis, dialysis into 50 mM NACL, 25 mM Tris HCL pH 8, 1 mM EDTA, 0. I% sodium azide, and 25 mM lactose, binding and elution from a P2 monoclonal antibody anti-RTB azlactone

functionality bis-acrylamide affinity matrix using 0. I M triethylamine HCL (pH 11) elution buffer and neutralization with 11 M NAPO, pH 4. Protein was quantitated by P2 antibody ELISA and reassociated overnight at room temperature with a three-fold molar excess of plant RTA (Inland Laboratories, Austin, TX). Heterodimer concentration was determined by a sandwich ELISA employing P2 monoclonal anti-RTB antibody capture and biotinylated αBR12 anti-RTA monoclonal antibody detection. Plant ricin was obtained from Inland Laboratories.

Glycosylation of W37S/Y78H/Y248H RTB. Using a previously reported procedure (53), two T-25 flasks were inoculated with 5 x 10^6 Sf9 cells, 10 ml of TMNFH media and recombinant baculovirus encoding RTB[W37S/Y78H/Y248H] at an moi of 1. After 48 hours, medium in one flask was changed to TMNFH plus 10 μg/ml tunicamycin. After 60 hours, the medium of both flasks was changed to Grace's medium without methionine supplemented with 10% dialyzed fetal bovine serum. Again, 10 μg/ml tunicamycin was added to one flask. After two hours, 250 μCi ^{35}S-methionine (1000 mci.mmol) was added to each flask. Four hours later, the medium was removed and replaced with PBS. One of the flasks again contained 10 μg/ml tunicamycin. After an additional six hours of incubation, the cells and supernatants from each flask were harvested. The cells were suspended in 150 mM NaCl/1% NP40/0.5% DOC/0.1% SDS/50 mM Tris pH 8/25 mM lactose (RIPAL). After freezing and thawing, cell extracts were centrifuged at 22,000g for 10 minutes. Pansorbin fixed *Staphyloccocus aureus* Cowan strain was prewashed with 3% BSA in PBS and then incubated with cell extracts to remove non-specifically-binding labeled materials. The metabolically-labeled solutions were then reacted with Pansorbin pretreated with rabbit anti-ricin antibody. The Pansorbin was then pelleted and washed with RIPAL twice and RIPAL plus 500 mM NaCl once. The pellets were then boiled in reducing

sample buffer and the samples run along with prestained low molecular standards on a 15% SDS polyacrylamide gel. The gel was fixed briefly with 10% acetic acid, soaked for one hour in Enlightening, dried, exposed to x-ray film with enhancing screens for 1-3 days at -70°C and developed.

Macrophage cells. Mouse peritoneal macrophages were prepared using 12 week old female Balb/c mice house in an IACUC approved facility. Briefly, mice were injected intraperitoneally with 1.5 ml Brewer's thioglycollate medium (Becton Dickinson Microbiology Systems). After five days, mice were sacrificed. The mice were then injected into their thigh fatty deposits with 10 ml of cold HEPES buffered Hank's balanced salt solution with 10 U/ml heparin with 11% BSA. After injection, the mice were gently shaken and the injected medium withdrawn. The cell suspension was centrifuged at 1,000x g for 10 minutes and the cell pellet resuspended in 10 ml of RPMI1640 with 10% fetal calf serum and 0.01 M HEPES. Cells were then diluted to 2 x 10^6/ ml and seeded to 96 well plates at 100 µl/well. After incubation for two hours at 37°C/5% CO_2, the plates were washed three times with warm Hank's balanced salt solution buffered with HEPES. After washing, 100 µl/well leucine-free RPMI1640 medium with 10% dialyzed fetal calf serum was added. 35 mm petri dishes were treated similarly except 2 ml of cells representing 2 x 10^5 cells/dish was used.

The J774E mouse macrophage cell line (56) was cultured in α-MEM with 10% fetal calf serum, 60 µ thioguanine and transferred from flasks to wells and dishes by exposure to trypsin EDTA (Gibco). Cells were plated at 2 x 10^4 cells/well in 96 well plates and 2 x 10^5 cells/35 mm dish, and incubated a further 24 hours at 37°C/5% $C0_2$ prior to assay.

MMR61 rat fibroblasts transfected with mouse mannose receptor cDNA were grown in Dulbecco's MEM containing 10% fetal calf serum and 400 ug/ml G418 (57). Cells were split and transferred to wells and dishes again by trypsinization and incubated a further 24 hours prior to assay.

KB human epidermoid carcinoma cells obtained from the American Type Culture Collection (Rockville, MD) were grown in Dulbecco's MEM with 10% fetal calf serum (58). Cells were removed from flasks by trypsin treatment and seeded for experiments identically to the other cell types.

Cytotoxicity assays. After two washes with Hank's balanced salt solution, the mouse peritoneal macrophage cells in each well of the 96 well plate were resuspended in 100 µl of leucine-free RPMI1640 with 10% dialyzed fetal calf serum and different concentrations of toxin. Twelve different concentrations of toxin were used in each experiment. Sets of wells in each experiment contained 60 mM lactose and/or 1 mg/ml yeast mannan. After 20 hours, 50 µl of leucine-free media containing 1 uCi ^{3}H-leucine (Amersham, 300 mCi/mmol) with or without added lactose and/or mannan was added to the wells. The cells were again incubated four hours at 37°C/5% $C0_2$ and then harvested with a Skatron cell harvester onto glass fiber filter mats. Filters were dried and counted in Econofluor liquid scintillation fluid in a LKB liquid scintillation counter. The IC_{50} was determined for each toxin/cell type/medium condition as the toxin concentration that reduced protein synthesis to 50% of control. Each assay was performed in quadruplicate. The mannose receptor directed toxicity was quantitated by the ratio of the toxin IC_{50} in the presence of lactose plus mannan to the IC_{50} in the presence of lactose alone. KB cells were assayed identically. J774E and MMR61 were assayed identically except leucine-free DMEM medium was used instead of RPMI1640.

Immunofluorescence assay. Mouse peritoneal macrophages, J774E, MMR61 and KB cells were attached for 24 hours at 37°C in 5%$C0_2$ to petri dishes, fixed in 3.7% formaldehyde in PBS (15 minutes), washed with 2 mg/ml BSA in PBS and 0.1% saponin and incubated in PBS plus BSA with rabbit anti-mouse mannose receptor antibody (56) for 30 minutes at 4°C. The cells were rewashed with PBS and reacted with goat anti-(rabbit Ig) conjugated to rhodamine (Jackson ImmunoResearch, West Grove, PA) at 25 ug/ml with 0.1% saponin for 30 minutes at 23°C. After a final wash with PBS, the cells were fixed in 3.7% formaldehyde in PBS, mounted under a #1 coverslip in glycerol-PBS (90:10) and examined using a Zeiss Axioplan epifluorescence microscope (63 x, N.A. 1.4 planapochromat objective). Fluorescence images were recorded using Tri-X film and negatives were digitized and edited using Adobe Photoshop software and a PowerMac 8500/120 computer. Relative intensities for the brightness of mannose receptor reactions in the different cell types were, respectively, for K-B, for J774E cells, and (+) for MMR61 and mouse peritoneal macrophages [scale (-) - (++++)].

Triple-site mutant RTB glycosylation. W37S/Y78H/Y248H RTB was immunoprecipitated from Sf9 infected cell extracts after metabolic labeling with ^{35}S-methionine in the presence and absence of tunicamycin.
Autoradiographs of reducing SDS polyacrylamide gels showed bands at 33 kilodaltons molecular weight in the absence of tunicamycin and 29 kilodaltons molecular weight in the presence of tunicamycin corresponding to 4 kilodaltons of attached sugars per molecule.

Triple-site mutant RTB-RTA heterodimer. Two preparations of W37S/Y78H/Y248H RTB-RTA were made each at 40 ug/ml concentration. Each showed 0. 1% asialofetuin binding avidity relative to plant ricin.

Mannose receptor-targeted ricin toxicity. Ricin-related proteins in the presence of excess lactose bind and intoxicate mannose receptor-deficient cells only weakly. The IC_{50} of ricin on KB cells in the presence of lactose was 3 x 10^{-9} M. The IC_{50} of the triple-site mutant RTB-RTA with lactose was 3 x 10^{-8} M.

In contrast, cells with mannose receptor are sensitive to ricin proteins even in the presence of excess lactose due to mannose-terminated oligosaccharides on plant and insect-derived ricin. The IC_{50}'s of ricin with lactose on mouse peritoneal macrophages, J774E cells and MMR61 rat fibroblasts transfected with mouse mannose receptor were 8 x 10^{-11} M, 1 x 10^{-10} M, and 8 x 10^{-10} M, respectively. Similarly, the triple-site mutant RTB-RTA with lactose showed IC_{50}'s on mouse peritoneal macrophages, J774E cells and MM61 cells of 1 x 10^{-9} M, 3 x 10^{-10} M, and 7 x 10^{-9} M, respectively. Thus, the receptor-positive cells were 4 - 36 fold more sensitive to ricin and 30-100 fold more sensitive to triple-site mutant RTB-RTA heterodimer than receptor-negative cells.

Lower efficacy of cell intoxication by mannose receptor pathway.

Ricin binds cell surface galactosides in the absence of lactose and intoxicates cells with an IC_{50} of 1 **X** 10^{-12}, 11 x 10^{-11} M and 2 x 10^{-11} M on mouse peritoneal macrophages, J774E cells and MMR61 cells, respectively. Thus, ricin was 10-80 fold more potent in the absence of lactose. The lectin-deficient mutant ricin had minimal residual galactoside mediated cytotoxicity with IC_{50}'S of 5 x 10^{-10} M, 7 x 10^{-10} M, and 3 x 10^{-9} M on mouse peritoneal macrophages, J774 E cells and MMR61 cells, respectively. This was a 0.3 - 2 fold relative potency to mutant heterodimer in the presence of lactose.

Competition of mannose receptor-mediated toxin uptake by mannan. The D-mannose receptor negative KB cells showed no inhibition of ricin cytotoxicity by mannan versus control (IC_{50} Of 9×10^{-12M} for both) or mannan plus lactose versus lactose alone (IC_{50} of 3×10^{-8}M for both). Similarly, KB cells showed no effects of mannan on lectin deficient ricin toxicity in the absence of lactose (IC_{50} of 1×10^{-8} M for both) or presence of lactose (IC_{50} of 3×10^{-8} M for both).

In contrast, ricin toxicity to all three mannose receptor positive cell lines in the presence of lactose was inhibited by mannan. Mannan increased the IC_{50} to 2×10^{-10} M, 3×10^{-9} M, and $3 \times {}^{10\text{-}9}$M for mouse peritoneal macrophages, J774 E cells, and MMR61 cells, respectively. This represented a 3 - 30 fold reduction in toxicity. Lectin-deficient ricin behaved like ricin with significant mannan inhibition of toxicity on these cell lines. Mannan increased the IC_{50} to 5×10^{-9} M, 2×10^{-8} M, and 3×10^{-8} M for mouse peritoneal macrophages, J774E cells and MMR61 cells, respectively. This yielded a 4 - 50 fold reduction in toxicity.

Immunologic detection of D-mannose receptor. Rabbit antibody to mouse mannose receptor reacted strongly with both surface and intracellular sites in J774E and MMR61 cells lines and many of the adherent cells from thioglycollate treated mouse peritoneal fluid cells. No binding was observed with the KB human epidermoid carcinoma cells. Localization was seen not only in an intracellular granular organelle pattern, but also in association with the ruffled border of cells, consistent with a cell surface distribution.

The mechanism by which internalized ricin toxin is transported to a translocation competent intracellular organelle is unknown. Plant ricin and insect-derived RTBRTA heterodimers have mannose-rich N-glycans and thus

may be bound and internalized by cells possessing the D-mannose receptor in the presence of excess lactose to block binding to cell surface galactosides. These studies demonstrate that RTB intracellular galactose binding activity is not required for ricin toxicity. Surprisingly, the lectin-deficient ricin retained cytotoxic potency implying either that RTB lectin activity is not required for intracellular transport and cell intoxication or that the small amount of residual lectin function (0.1% of normal) is sufficient for critical intracellular routing.

The lower potency of mannose receptor-mediated toxicity for both lactose blocked ricin and lectin-deficient ricin may be due to reduced surface receptor content or lower avidity of cell surface binding for the D-mannose receptors and may not reflect altered intracellular processing. The number of galactosyl-terminated cell surface glycoprotein receptors for ricin has been reported to be about 10^7/mammalian cell (59) versus 10^5/cell for D-mannose receptors (56). Avidities for each were similar with Ka's of 10^9 M^{-1} (56,59).

The residual cell toxicity of ricin-related molecules in the presence of mannose and lactose was 10^{-9} - 10^{-8} M. This toxicity is ten-fold higher than RTA alone and probably reflects incomplete competition at 37°C by the soluble lactose and yeast mannan.

The greater sensitivity of J774E cells relative to MMR61 or mouse peritoneal macrophages may be due to higher cell surface mannose receptor density on J774E cells or different intracellular metabolism. The immunofluorescence assay suggests higher receptor content is the cause for the J774E sensitivity difference.

The observation of efficient cell killing in the absence of RTB lectin function for mannose receptor has also been documented for the IL2 receptor

using an insect-derived lectin-deficient IL2 ricin fusion molecule. RTB enhancement for other receptors may be due to inefficient internalization by the ligand-receptor complex or misrouting intracellularly away from a translocation competent compartment (61,62).

Exploration of the intracellular steps in ricin intoxication remains an important avenue for defining molecular signals for routing of soluble intracellular polypeptides. This study provides a model system for examining other ligand-receptors and combined with the new molecular tags such as green fluorescent protein (63), may permit better understanding of important chemical reactions that mediate vectorial transport inside cells.

Throughout this application, various publications are referenced. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

Although the present invention has been described with reference to specific details of certain embodiments thereof, it is not intended that such details should be regarded as limitations upon the scope of the invention except as and to the extent that they are included in the accompanying claims.

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Table 1. Properties of insect-derived ricin proteins*

Protein	Galactose binding (kD asialofetuin)	Cell cytotoxicity (IC_{50} HUT102 cells)
Ricin	4×10^{-9} M	4×10^{-12} M
RTB[Y248H]-RTA	2×10^{-8} M	8×10^{-11} M
RTB[W37S/Y248H]-RTA	2×10^{-7}M	2×10^{-10} M
RTB[W37S/Y78H/Y248H]-RTA	6×10^{-6}M	5×10^{-9} M
RTA	$> 10^{-5}$ M	1×10^{-7}M

*Galactose binding quantitated based on RTB binding to immobilized asialofetuin. Cell cytotoxicity measured by 20 hour exposure to toxin in leucine-free media followed by 4 hour pulse with ^{3}H-leucine and filtration on glass fibre mats. Ricin and RTA obtained from castor beans.

Table 2. Cell cytotoxicity of ricin fusion proteins

Cell line	IC_{50}(M)			
	RICIN	IL2-W.T. RICIN	IL2-D.S. RICIN	IL2-T.S. RICIN
HUT102	2×10^{-12}	2×10^{-12}	4×10^{-12}	5×10^{-12}
CEM	3.5×10^{-12}	4×10^{-12}	1.8×10^{-10}	1×10^{-9}
KB	2×10^{-12}	2×10^{-12}	2×10^{-10}	6×10^{-10}
MT-1	2×10^{-12}	3×10^{-12}	1×10^{-11}	8×10^{-11}
YT2C2	2×10^{-12}	4×10^{-12}	9×10^{-12}	1×10^{-11}

*Cell cytotoxicity assays performed in triplicate as described in text. IC_{50} is the concentration of toxin reducing protein synthesis by 50% after 24 hour incubation. IL2-W.T. RICIN= IL2-wild-type RTB-RTA; IL2-D.S. RICIN=IL2-RTB[W37S/Y248H]-RTA; IL2-T.S. RICIN=IL2-RTB[W37S/Y248H/Y78H]-RTA.

What is claimed is:

1. A plant holotoxin fusion protein comprising a plant holotoxin comprising 1α, 1β and 2γ subdomains and having a modification in a lectin binding site in each of the 1α, 1β and 2γ subdomains and a ligand specific for a cell surface receptor.

2. The fusion protein of claim 1, wherein the modification in a lectin binding site in each of the 1α, 1β and 2γ subdomains consists of an amino acid substitution.

3. The fusion protein of claim 2, wherein the amino acid substitution is a substitution of an amino acid having an aromatic ring with an amino acid lacking an aromatic ring.

4. A ricin toxin B chain fusion protein comprising a ricin toxin B chain having a modification in a lectin binding site in each of the 1α, 1β and 2γ subdomains and a ligand specific for a cell surface receptor.

5. The fusion protein of claim 4, wherein the modification in a lectin binding site in each of the 1α, 1β and 2γ subdomains consists of an amino acid substitution.

6. The fusion protein of claim 5, wherein the amino acid substitution is a substitution of an amino acid having an aromatic ring with an amino acid lacking an aromatic ring.

7. The fusion protein of claim 6, wherein the amino acid substitutions consist of a W to S substitution at amino acid position 37 in the 1α subdomain, a Y to H substitution at amino acid position 248 in the 1β subdomain and a Y to H substitution at position 78 in the 2γ subdomain.

8. The fusion protein of claim 4, wherein the ligand is interleukin-2.

9. The fusion protein of claim 4, wherein the ligand is granulocyte/macrophage colony stimulating factor.

10. The fusion protein of claim 4, wherein the ligand is an antibody to CD3.

11. The fusion protein of claim 4, wherein the ligand is an antibody to GD2.

12. The fusion protein of claim 4, wherein the ligand is epidermal growth factor.

13. A ricin toxin B chain fusion protein comprising a moiety consisting of a ricin toxin B chain comprising a W to S substitution at amino acid position 37 in the 1α subdomain, a Y to H substitution at amino acid position 248 in the 1β subdomain and a Y to H substitution at position 78 in the 2γ subdomain and a moiety consisting of a ligand specific for a cell surface receptor.

14. The fusion protein of claim 13, wherein the ligand is interleukin-2.

15. The fusion protein of claim 13, wherein the ligand is granulocyte/macrophage colony stimulating factor.

16. The fusion protein of claim 13, wherein the ligand is an antibody to CD3.

17. The fusion protein of claim 13, wherein the ligand is an antibody to GD2.

18. The fusion protein of claim 13, wherein the ligand is epidermal growth factor.

19. A ricin fusion protein immunotoxin comprising the ricin toxin B chain fusion protein of claim 13 associated with a ricin toxin A chain.

20. The ricin fusion protein immunotoxin of claim 19 in a pharmaceutically acceptable carrier.

21. A nucleic acid encoding the fusion protein of claim 1.

22. A vector comprising the nucleic acid of claim 21.

23. A host comprising the vector of claim 22.

24. A nucleic acid encoding the fusion protein of claim 4.

25. A vector comprising the nucleic acid of claim 24.

26. A host comprising the vector of claim 25.

27. A plant holotoxin comprising 1α, 1β and 2γ subdomains and having a modification in a lectin binding site in each of the 1α, 1β and 2γ subdomains.

28. The plant holotoxin of claim 27, having at least a one thousand fold reduction in sugar binding and at least a one hundred fold reduction in toxicity in mice.

29. The plant holotoxin of claim 27, wherein the modification in a lectin binding site in each of the 1α, 1β and 2γ subdomains is an amino acid substitution.

30. The plant holotoxin of claim 29, wherein the amino acid substitution consists of substitution of an amino acid having an aromatic ring residue with an amino acid lacking an aromatic ring residue.

31. A ricin toxin B chain having a modification in a lectin binding site in each of the 1α, 1β and 2γ subdomains.

32. The ricin toxin B chain of claim 31, having at least a one thousand fold reduction in sugar binding, associating normally with ricin A toxin and having at least a one hundred fold reduction in toxicity in mice.

33. The ricin toxin B chain of claim 31, wherein the modification in a lectin binding site in each of the 1α, 1β and 2γ subdomains is an amino acid substitution.

34. The ricin toxin B chain of claim 33, wherein the amino acid substitution consists of substitution of an amino acid having an aromatic ring residue with an amino acid lacking an aromatic ring residue.

35. The ricin toxin B chain of claim 34, wherein the amino acid substitutions consist of a W to S substitution at amino acid position 37 in the 1α subdomain, a Y to H substitution at amino acid position 248 in the 1β subdomain and a Y to H substitution at position 78 in the 2γ subdomain.

36. A ricin fusion protein immunotoxin comprising the ricin toxin B chain fusion protein of claim 4 associated with a ricin toxin A chain.

37. The ricin fusion protein immunotoxin of claim 36 in a pharmaceutically acceptable carrier.

38. A nucleic acid encoding the plant holotoxin of claim 27.

39. A vector comprising the nucleic acid of claim 38.

40. A host comprising the vector of claim 39.

41. A nucleic acid encoding the ricin toxin B chain of claim 31.

42. A vector comprising the nucleic acid of claim 41.

43. A host comprising the vector of claim 42.

44. A method of constructing a ricin fusion protein immunotoxin comprising:

a) expressing the nucleic acid in the vector of claim 25 in a eukaryotic cell expression system to produce a fusion protein;

b) isolating and purifying the fusion protein of step a); and

c) contacting the fusion protein of step b) with a ricin toxin A chain under conditions which permit the association of the fusion protein with the ricin toxin A chain.

45. A method of treating a cancer or an autoimmune disease in a patient diagnosed with a cancer or an autoimmune disease comprising:

a) constructing the ricin fusion protein immunotoxin of claim 44, wherein the ligand is specific for a particular cell surface receptor present only on the surfaces of the cancer cells or on the surfaces of the cells causing the patient's autoimmune disease; and

b) administering the ricin fusion protein immunotoxin in a pharmaceutically acceptable carrier to the patient, whereby the ricin fusion protein immunotoxin treats the patient's cancer or autoimmune disease.

46. The method of claim 45, wherein the cancer is a human leukemia or lymphoma having cancer cells expressing interleukin-2 receptors on the surfaces and the ligand is interleukin-2.

47. The method of claim 45, wherein the autoimmune disease is graft-versus-host disease and the ligand is an antibody to CD3.

48. The method of claim 45, wherein the cancer is acute myelogenous leukemia and the ligand is granulocyte/macrophage-colony stimulating factor.

49. The method of claim 45, wherein the cancer is melanoma/neuroblastoma and the ligand is an antibody to GD2.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 97/19577

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K14/415 C12N15/62 A61K38/16

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 89 04839 A (GENETICS INST) 1 June 1989 see the whole document ---	1-49
X	FRANKEL A. ET AL.,: "Double-site ricin B chain mutants retain galactose binding" PROTEIN ENGINEERING, vol. 9, no. 4, - April 1996 pages 371-379, XP002059508 see whole document, esp. discussion ---	1-7,13, 19-45
Y	FRANKEL A. ET AL.,: "IL-2 ricin fusion toxin is selectively cytotoxic in vitro to IL2 receptor-bearing tumor cells" BIOCONJ. CHEM., vol. 6, no. 6, - 1995 pages 666-672, XP002059509 cited in the application see whole document, esp. discussion ---	1-8,13, 14,19-46
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 March 1998	08.04.98
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	**Authorized officer** Müller, F

1

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 97/19577

C.(Continuation) DOCUMENTS CONSIDERED T BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 89 01037 A (CETUS CORP) 9 February 1989 see claims and page 11, line 32 ff.	1-8,13, 14,19-46
A	SPHYRIS N. ET AL.,: "Mutational analysis of the ricin lectin B-chains" J. BIOL. CHEM., vol. 270, no. 35, - 1 September 1995 pages 20292-20297, XP002059510 see the whole document	1-49
A	RUTENBER E. & ROBERTUS J. D. : "Structure of Ricin B-chain at 2.5A resolution" PROTEINS, vol. 10, - 1991 pages 260-269, XP002059515 see the whole document	1-49
A	WILLIAMS D. P. ET AL.,: "Diptheria toxin receptor binding domain substitution with interleukin-2: genetic construction and properties of a diphteria toxin-related interleukin-2 fusion protein" PROTEIN ENGINEERING, vol. 1, no. 6, - 1987 pages 493-498, XP002059511 see the whole document	1-49
P,X	FRANKEL A. E. ET AL.,: "IL2 fused to lectin-deficient ricin is toxic to human leukemia cells expressing the IL2 receptor" LEUKEMIA, vol. 11, - January 1997 pages 22-30, XP002059512 see the whole document	1-8,13, 14,19-46
P,X	BURBAGE C. ET AL.,: "Rcin fusion toxin targeted to the human granulocyte-macrophage colony stimulating factor receptor is selectively toxic to acute myeloid leukemia cells" LEUKEMIA RESEARCH, vol. 21, no. 7, - July 1997 pages 681-690, XP002059513 see the whole document	1-7,9, 13,15, 19-45,48
P,X	FRANKEL A. E. ET AL.,: "Ricin toxin contains at least three galactose-binding sites located in B chain subdomains 1alpha,1beta, and 2gamma" BIOCHEMISTRY, vol. 35, - 26 November 1996 pages 14749-14756, XP002059514 see the whole document	1-8,13, 14,19-46

1

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 97/19577

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 45-49
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 45-49 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 97/19577

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 8904839 A	01-06-89	EP 0341304 A	15-11-89
		JP 2502287 T	26-07-90
WO 8901037 A	09-02-89	AU 2136788 A	01-03-89

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